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EXPERIMENTAL STUDIES ON THE ETIOLOGY OF TYPHUS FEVER.

VI. Skin Lesions in Experimental Typhus Fever of Guinea Pigs.

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, October 8, 1923.)

In a previous article[1] mention was made of specific vascular and nodular lesions in the corium of the skin in experimental typhus fever of guinea pigs. With a few exceptions, among whom are Löwy[2] and Wolbach,[3] other workers in this field, notably Otto and Winkler,[4] Otto and Dietrich,[5] and Neill,[6] have failed to find the lesions in the experimental disease. Hence no agreement on their presence in infected guinea pigs has been reached. Their recognition would be less difficult if the skin lesions could be intensified and consequently be made to appear in the form of an exanthem. This procedure, furthermore, would furnish another means for the precise definition of experimental typhus in the guinea pig. A further advantage would be the ease with which examinations for histopathology could be performed by merely excising the exanthematous areas. In this way, also, the possible incitant of the lesions might be studied. A number of tests could then be made at different periods of the fever without killing the animal. Finally, a cutaneous rash would be an additional indication of the identity of the experimental disease with typhus fever in man.

[1] Olitsky, P. K., *J. Exp. Med.*, 1921, xxxiv, 365.

[2] Löwy, O., *Wien. klin. Woch.*, 1916, xxix, 547.

[3] Wolbach, S. B., Todd, J. L., and Palfrey, F. W., The etiology and pathology of typhus, Cambridge, 1922, 195.

[4] Otto, R., and Winkler, F., *Z. Hyg. u. Infectionskrankh.*, 1921, xciii, 1.

[5] Otto, R., and Dietrich, *Centr. Bakt., 1. Abt., Orig.*, 1918, lxxxii, 383.

[6] Neill, M. H., *Pub. Health Rep., U. S. P. H.*, 1917, xxxii, 1105.

In this paper the exanthematous reaction which the typhus virus produces in guinea pigs will be demonstrated by a method suggested by the experiments of Rivers and Tillett[7] in transmitting the chicken-pox virus from man to rabbits.

Methods.

The method consisted, in brief, of irritating a limited area of the skin of a guinea pig before the onset of the febrile or active stage of the disease, thus localizing the effects to the particular area. We sought for a mild irritant such as would not produce a dermatitis that would be confusing. After experimenting with a number of irritants, as ether, chloroform, adhesive plaster, mustard paste, and tar, applied to the shaved skin, and with depilation by means of calcium sulfide, we adopted finally simple shaving as the most satisfactory procedure.

The method employed for the demonstration of the exanthem was to select a white animal or one with an abdomen free from pigment and to clip the hair of the entire anterior abdominal wall with a No. 00 clipper. After lathering freely with ordinary white, alkaline, hand or toilet soap, we shaved this portion closely. In most instances the shaving was repeated on alternate days to clear the area of rapidly growing hair. The abdominal wall was prepared in this way on the 9th or 10th day after the inoculation with the typhus virus, or about 1 day before the onset of fever. A normal, uninoculated guinea pig was similarly treated in each experiment as a control.

Thus the normal appearance of the skin can be preserved so that an exanthem or other changes may be noted. However, by a more active or destructive irritation, a dermatitis is induced usually in the form of a hyperkeratosis. The inflammation then obscures any exanthem, but the histopathological lesions in the deeper layers of the skin become thereby more pronounced and numerous. A satisfactory procedure to effect this more severe dermal reaction consists of rubbing the skin briskly for a few seconds with ether or chloroform immediately after shaving, and once each day thereafter.

The typhus virus, in the form of defibrinated blood, or of saline solution suspensions of cerebral or of splenic tissue, was inoculated intraperitoneally, under chloroform anesthesia, in more than 40 guinea pigs, of which all showed the histological or exanthematous lesions to be described. The virus was originally derived from the blood of a typhus fever patient[1] and was in at least its 100th animal passage when the following observations were made.

Effects on the Skin.

On the 1st day of the experimental fever in guinea pigs, the skin shows a peculiar hyperemic redness which spreads over the entire

[7] Rivers, T. M., and Tillett, W. S., *J. Exp. Med.*, 1923, xxxviii, 673.

shaved area. This darkening of the natural tint persists for about 3 days; thereafter the normal color is restored. On the 2nd, but more often on the 3rd day of the fever, a distinct exanthem is noted, usually along the lateral areas and groin of the prepared portion of the skin. The eruption appears first as faint, hyperemic rose spots from pinpoint size to 1.5 mm. in diameter. These are few in number, scattered, and fade on pressure. Within a few hours, they become macular and distinctly petechial; the color changes to copper-red. They do not completely disappear on pressure at this stage, as the center always remains visible. The number of these faint macules increases so that on the 4th day of fever about 25 or 35 distinct lesions may be seen in an area 7 by 6 cm. They persist throughout the febrile stage, fading however, as the defervescence is reached. In some animals a faded exanthem is evident on the 1st or 2nd day of convalescence. In one instance, in addition to the rash, there was noted a purplish diffuse hemorrhagic area measuring 1 by 0.5 cm.

With guinea pigs when the more intensive method of irritation is employed, the resultant hyperkeratosis obscures the exanthem. In these animals, however, the histopathological lesions in the deeper layers of the skin are more pronounced and numerous than those which occur in the corium of infected guinea pigs whose epidermis has either not been treated or but mildly irritated.

The histopathology of the lesions in the corium has already been described[1] as consisting of nodular areas similar to those present in the brain, testicle, heart, and other organs. These nodules are composed mainly of endothelial leucocytes, or monocytes, together with a few leucocytes of the mononuclear and polymorphonuclear types. They are always to be found in proximity to the capillaries and arterioles. The endothelium of the vessels in certain areas is swollen and necrotic and the vascular lumina may contain thrombi. Surrounding the vessels, a collar of one or more layers of monocytes is seen. In addition, there are small and localized hemorrhages.

Microscopically the macular spots consist of an exudation of serum with formation of small vesicles beneath the cornified layer and a proliferation of monocytes in the stratified epithelium. Hemorrhages into the epidermal layers and necrosis of the epithelial cells are also manifest. In instances in which more intensive methods of irritation

are employed, these lesions, under a much thickened cornified layer, are more marked and extensive.

CONCLUSIONS.

The skin of guinea pigs in which the virus of typhus fever is propagated, when mildly irritated in advance of the febrile reaction, shows a characteristic exanthem during the height of the experimental disease. More drastic methods of irritation, however, cause a dermatitis which obscures the rash but produce in the corium more marked specific histopathological changes. The exanthem may aid the study of the specific incitant of typhus fever in the lesions.

GROWTH-INHIBITORY SUBSTANCES IN PNEUMOCOCCUS CULTURES.

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(Received for publication, September 25, 1923.)

INTRODUCTION.

In the study of bacterial symbiosis and antagonism, considerable interest has centered in the presence or absence of growth when organisms are reinoculated into filtrates of cultures of the same or different species. The nature of the substances which favor or inhibit the simultaneous or subsequent development of bacteria in the same nutritive medium has provoked much discussion and stimulated considerable research. The inhibitory influence which different microorganisms exert on one another has been ascribed, for the most part, to an altered and unfavorable reaction brought about in the substrate by growth, or to exhaustion of the medium by the dominant species, these two factors either singly or together resulting in autoinhibition or in the suppression of the more susceptible variety. In addition, it has been assumed that certain deleterious waste products accumulate as a result of bacterial metabolism which react unfavorably on subsequent growth, or even cause mutual inhibition of both the homologous and heterologous organisms.

In the case of pneumococcus it is known that the reaction of the medium is of great importance (1, 2). It has been shown that growth cannot be initiated in plain broth more acid than pH 7, or more alkaline than pH 8.3, and that the optimum reaction for growth is pH 7.8. When pneumococcus is grown in broth containing a readily fermentable sugar, as dextrose, the reaction becomes more acid, due, in part at least, to the fermentation of the sugar. When the accumulation of acid has proceeded to the point pH 5, growth ceases. However, if the filtrate of a dextrose broth culture is readjusted to pH 7.8, and again seeded with pneumococci, growth will occur. It is evident, then, that the production of increased acidity is sufficient, in itself, to inhibit growth eventually. Acid production is, therefore, one factor in the autoinhibition of growth. On the other hand, when bacteria-

free filtrates of plain broth cultures are readjusted to the optimum reaction for growth (pH 7.8) and reseeded with pneumococcus, no growth occurs. If, however, to this same filtrate a small amount of sugar, as dextrose, is added, multiplication again occurs. Moreover, it has been found in the course of the present study that when a mixture of one part of broth and nine parts of pneumococcus culture filtrate is reseeded with pneumococcus, the organisms will grow. The addition, then, of 10 per cent of unmetabolized or "fresh" broth to a pneumococcus culture filtrate suffices to restore to the exhausted medium some substance essential for growth. Since this reactivation of a culture filtrate is effected by the addition of dextrose as well as by plain broth, this action is evidently due to the presence of some readily utilizable substance, possibly of the nature of a carbohydrate. These facts indicate that in addition to acid production, still another factor is concerned in the mechanism of autoinhibition; namely, the exhaustion of nutritive material in the medium.

Certain active substances contained in the cell bodies of pneumococci play an important rôle in the metabolism of these organisms. It has been shown that, in addition to the endohemotoxin, described by Cole (3), pneumococci contain a group of intracellular enzymes which hydrolyze peptones, split fats, and cause hydrolysis of saccharose, starch, and inulin (4–6). The action of these intracellular enzymes of pneumococci leads to the accumulation of metabolic products in the culture fluid, which may inhibit further growth. The action of the proteolytic enzyme of pneumococcus upon the peptone of the culture fluid results in the production of amino acids (7). The more active the growth of the culture, within certain limits, the greater is the concentration of amino acids in the culture fluid. The action of esterase, a lipolytic enzyme of pneumococcus, results in the breaking down of the esters to fatty acids. Finally, the carbohydrate-splitting enzymes are known to bring about the hydrolysis of such substances as sucrose, starch, and inulin to simpler sugars; the subsequent fermentation of these sugars results in the production of acid.

Recently attention has been directed to a hitherto unrecognized inhibitory product of pneumococcus growth, which accumulates in media under certain cultural conditions. This substance is a peroxide. In 1921, McLeod and Govenlock (8) reported the occurrence of a heat-labile substance in pneumococcus cultures which inhibited the further growth of this and other organisms. This substance was later identified as hydrogen peroxide by McLeod and Gordon[1] (9, 10).

[1] The evidence presented by these investigators is strongly in favor of the view that this substance is hydrogen peroxide rather than some organic peroxide. In the present work no attempt to determine the chemical identity of this peroxide has been made beyond the observations that it reacts with benzidine in the presence of a catalyst and that it is heat-labile and decomposed by the oxidases of plant tissue.

To the action of this compound they ascribe the pigment changes brought about in blood by pneumococcus. These observers found that hydrogen peroxide is also formed by other bacteria of the lactic acid group.

In a preceding paper (11), it has been shown that under favorable conditions peroxide appears in broth cultures of pneumococcus during the logarithmic phase of growth and that it persists for many days. The factors favoring peroxide production and accumulation are abundant oxygen supply and the absence of catalase. The peroxide behaves in many ways like hydrogen peroxide. It is unstable, sensitive to heat, and decomposes rapidly in alkaline solutions. Under certain conditions peroxide-containing filtrates of pneumococcus cultures possess the further property of converting oxyhemoglobin into methemoglobin.[1]

In the present paper are recorded the facts thus far ascertained in the study of the inhibition of growth of homologous and heterologous organisms by the peroxide formed by pneumococcus; and certain other factors concerned in the mechanism of growth inhibition of pneumococcus are discussed.

EXPERIMENTAL.

The accumulation of acid and the exhaustion of the medium are conditions brought about by growth of pneumococcus. Either of these conditions may so alter the medium as to render it unsuitable for further growth. The study of the inhibitory effect of hydrogen peroxide upon growth must, therefore, be approached indirectly so that its action may not be confused with that of other inhibitory factors which may develop simultaneously in the medium. This was done by making a parallel study of the effect of hydrogen peroxide upon the growth of *Staphylococcus aureus*, an organism not so susceptible as pneumococcus to the acid and exhaustion factors of growth inhibition. The growth of pneumococcus and *Staphylococcus aureus* in broth and in pneumococcus culture filtrates containing peroxide was therefore observed.

The Inhibitory Action of Commercial Hydrogen Peroxide on Growth of Staphylococcus aureus and Pneumococcus.

The effect upon growth of the addition of small amounts of commercial hydrogen peroxide[2] to broth was first investigated.

Experiment 1.—Varying quantities of a 1:100 solution of commercial hydrogen peroxide were added to known amounts of plain broth. The concentration of peroxide in the broth ranged from 1:300 to 1:1,500. Each dilution was tested in the manner described[3] for the presence of hydrogen peroxide. 5 cc. portions of the broth containing hydrogen peroxide in varying dilutions were tubed and seeded with one loop of an 8 hour broth culture of *Staphylococcus aureus*. A similar series was also inoculated with 0.05 cc. of an 8 hour broth culture of Pneumococcus Type II. The cultures were incubated at 37°C. and were observed for the presence or absence of macroscopic growth at the end of 24 and 48 hours. The results are given in Table I.

In Table I it is seen that 3 per cent hydrogen peroxide in plain broth, in dilutions of 1:1,200, inhibits the growth of *Staphylococcus aureus* for 24 hours. In concentrations of hydrogen peroxide of 1:1,000 or greater, no growth of *Staphylococcus aureus* occurred within the 48 hour period of observation. The inhibitory effect of hydrogen peroxide upon growth of pneumococcus in plain broth is not so pronounced. 3 per cent hydrogen peroxide in dilutions 1:400 inhibits the growth of pneumococcus for 24 hours. In concentrations of 1:300 no growth of pneumococcus occurred.

Since the inhibition of growth of *Staphylococcus aureus* and of pneumococcus in filtrates of pneumococcus cultures was to be investigated, and this with reference to the presence of preformed peroxide in the culture filtrates, it seemed advisable to determine the effect of adding known quantities of commercial hydrogen peroxide to filtrates of pneumococcus cultures in which peroxide had not formed. This was done in Experiment 2.

[2] Hydrogen peroxide Merck, U.S.P. IX, was the reagent used in all experiments. On titration this preparation contained 3 per cent hydrogen peroxide.

[3] Peroxide test: 0.5 cc. of fluid to be tested is placed in an agglutination tube containing a piece of fresh plant tissue (potato). 2 drops of freshly prepared saturated solution of benzidine in glacial acetic acid are added. The appearance of a blue color on the potato or in the surrounding fluid indicates the presence of peroxide (11).

Experiment 2.—150 cc. of broth were seeded with 0.05 cc. of an 8 hour plain broth culture of Pneumococcus Type II. Owing to the cultural conditions which limited the free access of air the accumulation of peroxide was inhibited so that at the end of 24 hours only a small amount could be detected. The culture was then passed through a Berkefeld filter. The filtrate was readjusted to pH 7.8. The readjusted filtrate was autoclaved to destroy the remaining trace of preformed peroxide. A 5 cc. fraction of this was seeded with 0.05 cc. of a broth culture of Pneumococcus Type II. To portions of the autoclaved filtrate varying amounts of 1:100 commercial (3 per cent) hydrogen peroxide were added. 5 cc. quantities of the filtrate containing these known concentrations of peroxide were then seeded with one loop of an 8 hour broth culture of *Staphylococcus aureus*.

TABLE I.

Inhibition of Growth of Staphylococcus aureus and Pneumococcus by Commercial Hydrogen Peroxide.

Dilution of 3 per cent H_2O_2 in broth.	Benzidine test for H_2O_2.	Growth of *Staphylococcus aureus*.		Growth of Pneumococcus Type II.	
		24 hrs.	48 hrs.	24 hrs.	48 hrs.
1:300	+	0	0	0	0
1:400	+	0	0	0	+
1:500	+	0	0	0	+
1:600	+	0	0	+	+
1:700	+	0	0	+	+
1:800	+	0	0	+	+
1:900	+	0	0	+	+
1:1,000	+	0	0	+	+
1:1,200	±	0	+	+	+
1:1,500	±	+	+	+	+
Broth without H_2O_2.	0	+	+	+	+

Frequent observations were made during incubation to determine the presence or absence of macroscopic evidence of growth. The results are given in Table II.

From Table II it is evident that pneumococcus failed to grow when seeded into the homologous culture filtrate, even in the absence of peroxide. The failure of growth of pneumococcus under these conditions is therefore dependent upon factors other than peroxide. These factors have been referred to previously in this paper. The presence of small amounts of commercial hydrogen peroxide prevents growth of *Staphylococcus aureus* when this organism is seeded into

filtrates of pneumococcus cultures. A filtrate containing as little as 1:1,750 of 3 per cent hydrogen peroxide will inhibit growth of *Staphylococcus aureus* for 48 hours. As was shown in Experiment 1, in which the same lot of broth, unmetabolized, was used, a concentration of 1:1,000 of 3 per cent hydrogen peroxide is reached before this growth-inhibitory action upon staphylococcus is evident. It seems likely that this difference between metabolized and unmetabolized broth is due to the absence of certain nutritive elements in the former.

From Experiments 1 and 2 it seems clear that even small amounts of commercial hydrogen peroxide, when added to plain broth or

TABLE II.

Inhibition of Growth of Staphylococcus aureus in Pneumococcus Culture Filtrates by Commercial Hydrogen Peroxide.

Dilution of 3 per cent H_2O_2 in filtrate.	Growth of Staphylococcus R.					Growth of Pneumococcus Type II.
	12 hrs.	24 hrs.	48 hrs.	72 hrs.	6 days.	
1:1,000	0	0	0	0	0	0
1:1,200	0	0	0	0	0	0
1:1,500	0	0	0	0	0	0
1:1,750	0	0	0	++	++++	0
1:2,000	0	0	+	++++	++++	0
1:2,400	0	0	+++	++++	++++	0
1:3,000	0	+	++++	++++	++++	0
1:4,000	0	++++	++++	++++	++++	0
1:6,800	++++	++++	++++	++++	++++	0
Filtrate without H_2O_2.	++++	++++	++++	++++	++++	0

filtrates of pneumococcus cultures, exert an inhibitory action upon the growth of *Staphylococcus aureus*. On the other hand, pneumococcus grows in the presence of considerably higher concentrations of commercial hydrogen peroxide in broth than does staphylococcus, but fails to grow when reseeded into homologous culture filtrates, even in the absence of peroxide.

The Inhibitory Action of Peroxide Formed by Pneumococcus upon Growth of Staphylococcus aureus and Pneumococcus.

In order to determine whether or not the peroxide which develops in cultures of pneumococcus is concerned in the phenomenon of in-

hibition of growth which occurs when the same or other organisms are seeded into pneumococcus culture filtrates, the following experiment was performed.

Experiment 3.—0.5 cc. of a 6 hour plain broth culture of Pneumococcus Type II was seeded into 450 cc. of broth contained in a large Erlenmeyer flask. At the end of 27 hours incubation, a portion of the culture fluid was passed through a Berkefeld filter. The filtrate was readjusted to pH 7.8. The peroxide test was found to be positive. The filtrate was then divided. One part was autoclaved (120°C. for 10 minutes) in order to destroy the peroxide, and the other part left unheated. 5 cc. portions of the autoclaved and unautoclaved filtrates were seeded with 6 hour plain broth cultures of Pneumococcus Type II and *Staphylococcus aureus*, respectively. The inoculum, in the case of the pneumococcus, was 0.05 cc., and in the case of the staphylococcus, one 2 mm. loop. The specimens were incubated and observed first after 10 hours incubation, and then daily for 5 days. The results are given in Table III.

TABLE III.

Inhibition of Growth of Staphylococcus aureus and Pneumococcus in Heated (120°C. for 10 Minutes) and Unheated Filtrates of Pneumococcus Cultures.

Organism.	Growth in unheated filtrate.		Growth in autoclaved filtrate.	
	10 hrs.	5 days.	10 hrs.	5 days.
*Staphylococcus aureus.**	0	0	++	++++
Pneumococcus Type II.*	0	0	0	0

*When 5 cc. tubes of broth were seeded with these organisms, the inoculum being that used in Experiment 3, abundant growth occurred in 10 hours in each instance. In the case of the staphylococcus, the broth culture was distinctly heavier than that obtained when the autoclaved filtrate was inoculated with this organism.

This experiment shows that when staphylococcus is seeded into the filtrate of a pneumococcus culture containing preformed peroxide, no growth occurs. However, if such a filtrate is autoclaved prior to inoculation so that the peroxide is destroyed, good growth of staphylococcus is obtained within 10 hours. On the other hand, as was shown in the previous experiment, when pneumococcus is reseeded into the filtrate of pneumococcus culture, little, if any, growth occurs, regardless of the presence or absence of peroxide.

If the inability of staphylococcus to grow in the filtrate of a pneumococcus culture is due to the presence of peroxide, those filtrates which contain no peroxide should support growth of this organism. The

following experiment with filtrates of cultures in which the organisms grew under conditions such that peroxide could not form, bears upon this point. A comparative study was made of the growth of *Staphylococcus aureus* in a peroxide-containing filtrate and in similar filtrates in which, however, the accumulation of peroxide was prevented by cultural conditions.

Experiment 4.—50 cc. of plain broth in a 250 cc. Erlenmeyer flask were inoculated with 0.25 cc. of an 8 hour culture of Pneumococcus Type II. Another flask containing 50 cc. of 1 per cent dextrose broth was similarly inoculated. These two flasks were incubated at 37°C. under aerobic conditions. A third flask containing 50 cc. of plain broth was inoculated with 0.25 cc. of an 8 hour culture of Pneumococcus Type II, and incubated at 37°C. under anaerobic conditions (Brown's modified anaerobic jar). At the end of 18 hours the three cultures were filtered through Berkefeld candles. Tests for peroxide were performed upon the filtrates. 5 cc. portions of each filtrate were then tubed and seeded with one loop of actively growing broth culture of *Staphylococcus aureus*. At the end of 18 hours incubation the presence or absence of growth was noted. The results are given in Table IV.

TABLE IV.

Inhibition of Growth of Staphylococcus aureus in Unheated Filtrates of Pneumococcus Cultures in the Presence and Absence of Peroxide.

5 cc. of unheated filtrate of Pneumococcus Type II culture.	Peroxide test after 18 hrs. at 37°C.	Growth of staphylococcus in 18 hrs.
Plain broth (aerobic culture).....	+	0
" " (anaerobic ").....	0	+
1 per cent dextrose broth (aerobic culture).....	0	+

The experiment shows that *Staphylococcus aureus* will grow in the unheated filtrates of pneumococcus cultures in which peroxide is not present in detectable amounts. In the filtrate of the anaerobic culture, in which the formation of peroxide was prevented by the exclusion of air, and in the filtrate of the dextrose broth culture, growth of *Staphylococcus aureus* occurred. On the other hand, in the filtrate of the aerobic cultures in which peroxide was demonstrated by the benzidine reaction, growth did not occur.

The Nature of the Inhibitory Action of Peroxide upon Staphylococcus.

The bacteriostatic influence of commercial hydrogen peroxide is strikingly shown in Experiment 2 (Table II). It is well known that

this substance is also bactericidal. In previous experiments the inhibitory action of the peroxide of bacterial origin has been demonstrated. It seemed of interest, therefore, to determine whether the peroxide formed by pneumococcus possesses bactericidal as well as bacteriostatic properties.

Experiment 5.—Pneumococcus Type II was grown in broth under conditions such that the ratio of surface area to total volume of culture fluid was large. These conditions were optimum for the production of peroxide (11). Fractions of the culture were removed after 13, 16, 19, and 27 hour periods of incubation, and passed through a Berkefeld filter. The filtrates thus obtained were readjusted to pH 7.8 by the addition of N/10 alkali. 5 cc. quantities of these filtrates containing peroxide were then inoculated with one loop of an actively growing broth

TABLE V.

The Bacteriostatic and Bactericidal Action on Staphylococcus aureus of the Peroxide of Pneumococcus Culture Filtrates.

Filtrate of pneumococcus culture after incubation at 37°C.	Peroxide test immediately after filtration.	Inoculum of *Staphylococcus aureus.*	Growth of *Staphylococcus aureus* after 7 days of incubation.	Peroxide test after 7 days of incubation.	Second inoculum of *Staphylococcus aureus.*	Growth of *Staphylococcus aureus*		
						18 hrs.	42 hrs.	96 hrs.
hrs.								
13	+	1 loop.	0	0	1 loop.	+	+	+
16	+	1 "	0	0	1 "	+	+	+
19	+	1 "	0	Faint +	1 "	0	0	+
27	+	1 "	0	"	1 "	0	+	+

culture of *Staphylococcus aureus*. At the end of 7 days there was no evidence of growth. And subcultures on blood agar showed that no viable organisms were present. In these filtrates, in which the staphylococcus had not only failed to grow, but had been killed, peroxide was still present in demonstrable amounts in two instances. It is of interest to note that the filtrates in which peroxide persisted over a period of 7 days represented a fraction of the original culture after longer periods of growth and in which consequently the concentration of peroxide was presumably greater. The absence of peroxide in the other two filtrates is attributable to the destruction of this substance in culture fluids. The factors influencing the decomposition of this substance in media have been referred to in a preceding paper (11). The four filtrates, in all of which *Staphylococcus aureus* had previously failed to grow, and in two of which traces of peroxide were still demonstrable, were a second time inoculated with the same strain of *Staphylococcus aureus*. Growth promptly occurred in the two filtrates in which peroxide had

disappeared. In the other two filtrates, in which traces of peroxide still persisted, growth was delayed, and occurred only after a considerable period of lag. The results are recorded in Table V.

Experiment 5 shows that the peroxide in pneumococcus filtrates is not only bacteriostatic for *Staphylococcus aureus*, *i.e.* prevents cell multiplication, but also bactericidal. That the peroxide is actually the inhibitory agent is further shown by the fact that two of these same filtrates which, during the 7 days of incubation, had become peroxide-free, supported prompt and abundant growth of staphylococcus when again inoculated with this organism. In the two instances in which peroxide still persisted a considerable period of lag preceded active growth.

DISCUSSION.

The original studies of McLeod and his associates on the production of hydrogen peroxide by bacteria, and the subsequent confirmation by Avery and Morgan of the factors influencing the formation of peroxide by pneumococcus have led to the present observations on the nature of the phenomenon of growth inhibition in culture filtrates of pneumococcus. In an earlier publication it was pointed out that bacteria-free filtrates of *plain* broth cultures of this organism, even though readjusted to the optimal reaction, will not again support growth of pneumococcus unless small amounts of sugar (dextrose) are added. On the other hand, it was found that filtrates of *dextrose* broth cultures of pneumococcus, under optimal conditions of reaction, apparently contain sufficient unutilized carbohydrate to permit growth on reinoculation of the medium. The ability of pneumococci to multiply in a medium in which they have previously grown, was at that time thought to be dependent, in part at least, upon the maintenance of a suitable reaction and upon the presence of a residuum of fermentable substance left unmetabolized by previous growth.

The knowledge that pneumococci form peroxide and that this compound can be demonstrated in culture fluids, adds another factor in explanation of the phenomenon of autoinhibition of growth. The diffusion into the medium of this inhibitory substance is sufficient, in some instances at least, to account for the inability of certain

organisms to grow in pneumococcus filtrates. As pointed out in the present paper, growth of staphylococcus cannot be initiated in peroxide-containing filtrates of pneumococcus cultures. Evidence that peroxide formed during the growth is itself solely responsible for the lack of the subsequent growth of *Staphylococcus aureus* is found in the fact that staphylococci will grow in these same culture fluids after the peroxide has been destroyed by heat or deteriorated through age. Furthermore, staphylococci grow abundantly in filtrates of pneumococcus cultures in which the formation of peroxide has been prevented. In the case of *Staphylococcus aureus* the presence of peroxide is, therefore, the determining factor in the inhibition of growth, and the other two factors already referred to, namely altered reaction and exhaustion of the medium, are little, if at all, concerned in the inhibitory mechanism. In the case of pneumococcus, however, growth inhibition of this organism in its own culture fluid is conditioned primarily by the exhaustion of nutritive material and by reaction changes in the medium, and only secondarily by the presence of peroxide, since in readjusted culture filtrates, even in the absence of peroxide, a second growth of pneumococci cannot be initiated unless some readily utilizable nutritive material is added.

CONCLUSIONS.

The known factors concerned in the autoinhibition of growth of pneumococcus are:

1. The accumulation of acid products of metabolism, resulting in unfavorable reaction changes in the medium.
2. The exhaustion of the nutritive substances of the medium.
3. Under certain cultural conditions the formation and accumulation of peroxide in the medium.

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STUDIES ON OXIDATION AND REDUCTION BY PNEUMOCOCCUS.

I. Production of Peroxide by Anaerobic Cultures of Pneumococcus on Exposure to Air under Conditions Not Permitting Active Growth.

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INTRODUCTION.

The previous studies by McLeod and Gordon (1) and by Avery and Morgan (2) on the production of peroxide by pneumococcus have dealt with the formation and accumulation of this substance during active aerobic growth. The present paper presents a study of the production of peroxide by cultures in which its formation has been prevented during active growth, and in which the subsequent production of this compound could be followed under conditions unsuitable for the initiation and maintenance of cell reproduction. The cultures of pneumococcus employed in the following experiments were grown anaerobically in order to obtain bacterial cells not previously exposed to the action of the peroxide which may accumulate in the culture fluid during aerobic growth. Anaerobiosis was utilized, therefore, simply as a means of obtaining peroxide-free cultures in which could be studied the influence of certain factors on the potential ability of the cells to form peroxide when subsequently exposed to molecular oxygen.

The Formation of Peroxide by Anaerobic Cultures of Pneumococcus on Exposure to Air.

It was first desired to determine whether cells which had been grown anaerobically, that is under conditions precluding the formation of peroxide, would form this substance when subsequently exposed to air.

Cultures of Pneumococcus Type II were grown under vaseline seal in Florence flasks filled to the neck with broth. These cultures were incubated at 37°C. for 15 to 24 hours, which represent periods considerably beyond the phase of active growth. The anaerobic cultures were then cooled to 15°C. in running water; 5 cc. portions of broth culture were removed and placed in flasks of 300 cc. capacity. The culture fluid was then thoroughly aerated by shaking the flask for various lengths of time at room temperature. At intervals the culture fluid was tested for the presence of peroxide.[1]

Experiments of this nature have definitely shown that the bacterial cells, which have been grown under conditions precluding peroxide production, rapidly form peroxide when shaken in the air. Under these conditions peroxide in many instances may be demonstrated at the end of 5 minutes aeration. That the peroxide thus formed does not represent the oxidation of a metabolite free in the culture fluid, is shown by the fact that the supernatant fluid of centrifuged young anaerobic cultures remains peroxide-free after long periods of shaking in the air.

Influence of Age of the Culture upon the Formation of Peroxide by Anaerobically Grown Pneumococci When Exposed to Air.

The following experiment was conducted to test the influence of age and condition of the cell upon the ability of anaerobically grown pneumococci to form peroxide on exposure to air.

Two Florence flasks were completely filled to the neck with freshly boiled broth, sealed with vaseline, and inoculated each with a different strain of Pneumococcus

[1] Peroxide tests were made as follows: 0.5 cc. of the liquid to be tested was placed in a small agglutination tube. A small piece of freshly cut potato was introduced to serve as peroxidase. The presence of peroxide was then detected by the addition of 2 drops of a saturated solution of benzidine in glacial acetic acid. This test gave faint but definite reactions with broth containing 0.002 per cent of H_2O_2. By maintaining constant all conditions of the test, some idea of the comparative amount of peroxide can be estimated by the intensity of the reaction when compared with broth to which known amounts of titrated H_2O_2 had been added immediately before testing. The peroxidase-benzidine test is not specific for H_2O_2 and positive reactions would probably be given by any peroxide which can be split by the peroxidases of the potato. However, the fact that the peroxide which finally accumulates in pneumococcus cultures is hydrogen peroxide, has been proved by McLeod and Gordon.

Type II. After 15 hours incubation at 37°C., 15 cc. portions of each culture were transferred to a number of narrow test-tubes and sealed with vaseline. These anaerobic cultures, which had received the same initial seeding, were then allowed to age at 37°C. After periods indicated in Table I, the anaerobic cultures, free from peroxide, were exposed to air[2] in 150 cc. Erlenmeyer flasks at room temperature in the dark. The aerated cultures were tested for the presence of peroxide at frequent intervals during the period of exposure to air. The results of this experiment are condensed in Table I.

TABLE I.

Influence of Age of Cells upon the Rate and Persistence of Peroxide Formation by Anaerobically Grown Pneumococcus on Exposure to Air.

Age of anaerobic culture before exposure to air. (Pneumococcus Type II.)	Peroxide formation after exposure to air at 20°C.			
	Time of appearance.		Persistence after 10 days exposure to air.	
	Strain R.	Strain G.	Strain R.	Strain G.
hrs.	*hrs.*	*min.*		
15	4	30	+*	+
days				
2	6	30	−	+
3	6	30	−	+
4	6	30	−	+
6	8	30	−	−
		hr.		
15	†	1	−	−
70	‡	1	−	−

* In this table − indicates negative peroxide reaction; +, positive peroxide reaction.

† No peroxide detected.

‡ Faint traces of peroxide after 1 hour's exposure to air; the presence of detectable amounts was very transient, and only negative tests were obtained after 2 hours.

[2] Unless otherwise stated, cultures were "exposed to air" under the following conditions. The peroxide-free anaerobic cultures were shaken vigorously with air for 5 minutes. After preliminary agitation the aerated cultures were further exposed to molecular oxygen by placing the fluid in shallow layers at room temperature in the dark. The actual rate of peroxide production under these conditions is limited by the diffusion of oxygen into the non-agitated liquid. However, the preliminary, violent agitation, the shallow layers of liquid exposed, and the maintenance of constant conditions throughout all experiments, tend to minimize this objection in comparative studies of any given series.

The second and third columns in the table record the length of time required for the various cultures to produce a detectable amount of peroxide when exposed to the air. Hence, these figures in these columns give information concerning the rate of peroxide production by cells of different ages.

Peroxide is not a stable substance in broth and, even in the absence of catalase, it is gradually destroyed in this medium. Hence, the length of time the peroxide persists in the above tests will depend upon the actual amount of peroxide produced and upon the ability of the cells to continue its formation. The data presented in the fourth and fifth columns, therefore, serve as an index of the relative amount of peroxide produced by the cells of different ages, and of their ability to continue the production of peroxide.

It is apparent (Table I) that the "age" of the cultures at the time when the cells are exposed to oxygen affects the rate with which peroxide is formed. With increasing age of the anaerobic cultures there tends to be a gradual decrease of the potential peroxide-forming power of the cells. Nevertheless, it is evident that cultures which have passed beyond the phase of active growth and in which cell death and autolysis are occurring are still able to form peroxide when exposed to air.

It was thought that the storage of the mature anaerobic culture at low temperature would slow down the processes involved in the "aging" of the cells, and that, consequently, cultures held at low temperature would exhibit a more gradual loss in their ability to form peroxide. To investigate this question, the above experiment was performed in duplicate series, in which 15 hour anaerobic cultures were "aged" at 2° and at 37°C. Contrary to the results anticipated, no differences were observed in the effect of these two temperatures upon the ability of the anaerobically grown cells to form peroxide when exposed to molecular oxygen.

Table I also shows that marked differences exist in the readiness with which peroxide is formed by cells of different strains of pneumococcus. The shorter time required for production of detectable traces of peroxide shows that this process is much more rapid in young cultures of Strain G, than in those of Strain R. This difference in activity of peroxide production increases with the age of the cells. Strain R shows a gradual diminution of peroxide formation; Strain G, on the other hand, not only formed larger amounts of peroxide but its activity is less influenced by age than is that of the other strain.

That the more pronounced peroxide formation by Strain G is not related to viability or longevity was demonstrated by the fewer number of colonies developing on subculture.

Peroxide Formation by Anaerobically Grown Pneumococci When Exposed to the Air at Different Temperatures.

The ability of anaerobically grown pneumococci to produce peroxide when exposed to the air at different temperatures was tested in the following experiment.

TABLE II.

Peroxide Formation by Anaerobically Grown Pneumococci When Exposed to Air at Different Temperatures.

Period of exposure to air.	Strain R exposed to air at.			Strain G exposed to air at.		
	2–4°C.	20°C.	37°C.	2–4°C.	20°C.	37°C.
min.						
0	−	−	−	−	−	−
30	−	−	−	−	+	+
hrs.						
1	−	−	+	−	+	+++
2	−	−	++	−	+++	+++
4	−	+	++	+	+++	+++
6 and 9	−	++	++	++	+++	+++
days						
1, 2, and 3	−	++	++	++	+++	+++
5	−	+	+	++	+++	+++
10	−	±	±	++	+++	+++

In Tables II to IV − indicates that no peroxide was detected; ±, faint peroxide reaction; +, weak peroxide reaction; ++, moderate peroxide reaction; +++, marked peroxide reaction; and ++++, strong peroxide reaction.

15 cc. portions of the anaerobic cultures used in the preceding experiment were transferred to narrow test-tubes and overlaid with vaseline. The sealed cultures were placed in water baths at 2°, 20°, and 37°C. After they were brought to the desired temperature, the peroxide-free cultures were transferred to 150 cc. Erlenmeyer flasks and exposed to air. The respective temperature conditions were maintained throughout the manipulations. Peroxide tests were made at frequent intervals, the results of which are given in Table II.

Analysis of Table II shows that the maximum capacity to form peroxide was exhibited by pneumococcus cells aerated at 37°C. The

peroxide production by Strain G is again more pronounced and its activity is also much more independent of temperature than is that of Strain R. When the exposure to oxygen is carried out at 20°C., differences between the two strains become more marked. In the case of Strain R, an oxygen exposure of 4 hours is necessary before the appearance of peroxide in the culture. At a temperature of 2–4°C. Strain G shows a marked diminution in rate of peroxide production, while in the case of Strain R no peroxide was detected at any time during an observation period of 10 days. The fact that peroxide is formed by Strain G at 2°C. is evidence that this process may proceed at temperatures incompatible with cell multiplication.

Peroxide Production by Anaerobic Cultures of Pneumococcus When Readjusted to Different pH and Exposed to the Air.

It was desired to test the ability of anaerobically grown pneumococci to produce peroxide in culture fluid which, prior to aeration, had been readjusted to different hydrogen ion concentrations.

A broth culture of Pneumococcus Type II (Strain G) was grown in plain broth under anaerobic conditions for 15 hours at 37°C. At the end of the period of incubation the culture fluid, containing the anaerobically grown cells, was readjusted to pH zones 4 to 8.5 by the addition of N HCl and N NaOH. Under these conditions the organisms were subjected to the influence of these various reaction changes for 20 minutes before aeration. The readjusted cultures were then transferred to 150 cc. Erlenmeyer flasks and exposed to the air at room temperature. Tests were made at different intervals during aeration to determine the time of appearance of peroxide. The results are given in Table III.

Cultures in which the formation of peroxide has been precluded by the exclusion of air during growth may be subjected to reaction changes covering a wide range of H ion concentration without loss of their ability to form peroxide when subsequently exposed to the action of molecular oxygen (Table III).

Although the maximum production of peroxide occurs between pH 6.3 and 8.5, small amounts of peroxide may be produced at reactions approaching pH 5.0. That peroxide does not continue to be produced at this reaction is indicated by its gradual disappearance from the medium. At reactions more acid than pH 5.0 (as pH 4.0), no peroxide was formed even after prolonged aeration.

As shown in previous experiments, peroxide may be produced in cultures in which active growth has ceased and at temperatures outside the range of growth possibility. Likewise, in the present experiment, it has been found that the pH zone within which peroxide can be formed is considerably extended both on the acid and alkaline side beyond that which limits growth. In fact, the formation of small amounts of peroxide at pH 5.0 indicates that this process can be initiated at a pH which marks the acid death-point of pneumococcus.

TABLE III.

Peroxide Formation by Anaerobic Cultures of Pneumococcus When Readjusted to Different pH and Exposed to the Air.

pH to which culture was readjusted.	Peroxide formation after exposure to air for.				
	0	30 min.	2 hrs.	1 day.	4 days.
4.0	−	−	−	−	−
5.0	−	+	++	±	−
6.3	−	+	+++	++++	++++
7.1	−	+	+++	++++	++++
8.4	−	+	+++	++++	++++

Formation of Peroxide by Heated Anaerobic Cultures of Pneumococcus on Exposure to Air.

The following experiment was planned to determine whether or not anaerobically grown pneumococci which have been heated at 50°C. for varying periods of time, can form peroxide when subsequently exposed to air.

A 15 hour anaerobic culture of pneumococcus (Strain G) was prepared as described before. 4 cc. portions of this culture were placed in agglutination tubes and sealed with vaseline. These were then heated in a water bath at a constant temperature of 50°C. At stated intervals, duplicate tubes were removed and immediately cooled to 15°C. The contents of these tubes were then placed in 100 cc. Erlenmeyer flasks and exposed to the air at room temperature in the dark. Sterility tests of the heated cultures were made by plating 0.1 cc. on blood agar and inoculating 0.2 cc. in blood broth. Results are given in Table IV.

It is evident (Table IV) that, in the case of the strain of pneumococcus studied, the properties of the cell upon which peroxide for-

mation depends are thermolabile, and are destroyed by exposure to 50°C. for 15 to 30 minutes. Cultures which had been heated for 10 minutes at 50°C., but which contained a few viable cells, still retained the property of forming peroxide, in appreciable quantities, when subsequently exposed to the air. After 15 minutes exposure to this temperature, no viable cells could be determined by cultural methods, although significant, but transient, traces of peroxide were demonstrable after aeration.

TABLE IV.

Formation of Peroxide by Heated Anaerobic Cultures of Pneumococcus on Exposure to Air.

Anaerobic cultures heated at 50°C.	Peroxide tests after exposure of cultures to air at 20°C.				Presence of viable cells in culture tests after heating.
	0	1 hr.	2 hrs.	24 hrs.	
Unheated.	−	+++	+++	+++	+
min.					
5	−	+	++	++	+
10	−	+	++	+	+
15	−	±	±	−	−
30	−	−	−	−	−
60	−	−	−	−	−

DISCUSSION.

When cultures of pneumococci are grown anaerobically, and subsequently exposed to air, peroxide can be readily detected in the culture fluid. The formation of peroxide under these conditions is prompt, the reactive substances of the cells in the presence of molecular oxygen giving rise to demonstrable amounts of this compound after brief aeration. Although the property of forming peroxide was exhibited by all pneumococci, certain variations in degree of this activity and in the susceptibility of this process to environmental conditions were observed among different strains. Moreover, the property of the bacterial cell, upon which the formation of peroxide depends, is to some extent affected by the length of time the culture is held under anaerobic conditions before the cells are brought into contact with air. However, cultures, in the absence of molecular oxygen, still retain potentially reactive substances only slightly

impaired by the processes of aging when compared with the marked cellular changes which accompany senescence. Cell disintegration and autolysis, which always mark the cessation of growth of pneumococcus, have but little effect upon the avidity with which the cellular substances react with oxygen to form peroxide.

Cultures grown anaerobically, and then subjected in the absence of air to reaction changes covering a wide range of H ion concentration, pH 5.0 to 8.5, still possess the property of forming peroxide when subsequently exposed to the action of molecular oxygen. This range of H ion concentration is much wider than that within which cell growth can be initiated, and even includes the acid zone in which cell death occurs. Although temperature influences the rate of peroxide production, this property is exhibited by cultures exposed to temperatures much lower than those compatible with cell reproduction.

SUMMARY.

1. Anaerobically grown pneumococci rapidly form peroxide upon exposure to molecular oxygen.

2. The peroxide-forming activity of pneumococci varies with different strains, and with the age of the cell.

3. Peroxide production by pneumococcus can proceed under conditions of reaction and temperature that do not permit active growth and multiplication of the cell.

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STUDIES ON OXIDATION AND REDUCTION BY PNEUMOCOCCUS.

II. The Production of Peroxide by Sterile Extracts of Pneumococcus.

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In the preceding paper (1) it was shown that pneumococci are able to form peroxide within pH zones and temperature limits, and under other conditions, that do not permit active cell growth and multiplication. The present paper is concerned with the production of peroxide by sterile extracts prepared by freezing and thawing the pneumococcus cell; most of these preparations were filtered through Berkefeld filters and all of them were proved sterile by cultural and animal tests. The following report, therefore, represents a study of peroxide production by sterile extracts of the cellular substances of pneumococcus entirely free from living and formed cells. In later papers, there will be reported other phenomena exhibited by the oxidation-reduction systems contained in these sterile extracts.

Peroxide Production by Bile Solutions of Pneumococcus.

The first attempts to study the formation of peroxide by pneumococcus extracts were made on bile solutions of the cells.

Broth cultures of Pneumococcus Type II (Strains R and G) were grown anaerobically. 0.5 cc. of sterile ox bile was added to 15 cc. of the anaerobic broth cultures in narrow tubes which were then sealed with vaseline and incubated at 37°C. for 12 hours. In some experiments, the bile-treated cultures were held several days in the ice box after incubation to bring about maximum solution of the cells. The clear, bacterial solutions were then centrifuged to remove any cells remaining undissolved. The supernatant fluid was removed from under seal and exposed to the air in Erlenmeyer flasks. Sterility tests were made by plating 0.1 cc. on blood agar and inoculating 0.2 cc. in blood broth.

Considerable difficulty was experienced in obtaining absolutely sterile preparations, although no difference in rapidity of peroxide formation was observed in solutions proving sterile and in those showing the presence of a few viable cells. The formation of peroxide could usually be detected after 20 to 30 minutes exposure of the bile solution of pneumococci to the air at room temperature. The peroxide, however, did not persist in the bile solution, apparently due to its rapid decomposition in this medium. Solutions giving positive peroxide reactions after aeration for 20 to 30 minutes, frequently became negative after 2 hours. Peroxide formation could be demonstrated in the case of bile solutions aerated at 2–4°C. although 2 to 3 hours aerobic exposure was required.

Although peroxide production was exhibited by the sterile bile solutions of pneumococcus, these did not lend themselves so readily to further study as did extracts prepared by repeating freezing and thawing of the cells.

Peroxide Production by Sterile, Filtered Cell Extracts of Pneumococcus.

Preparation of Extracts.—The method of preparing these extracts was as follows:

Anaerobic broth cultures of Pneumococcus Type II were centrifuged and the unwashed cells suspended in small quantities of the supernatant broth or in sterile phosphate solutions so that 1 cc. of the resultant suspension contained the bacteria from 35 cc. of culture, the actual concentration varying in different preparations. The suspensions of unwashed bacteria were then alternately and rapidly frozen and thawed and this process repeated six to nine times until maximum disruption of the cells occurred. Anaerobic conditions were maintained during the freezing and thawing process, by keeping the cell suspensions in long narrow tubes under vaseline seal. After the freezing process the suspensions were centrifuged 1 hour at high speed. The amorphous, Gram-negative detritus removed by the first centrifugation was practically devoid of formed cells. The supernatant fluid was removed as free as possible from cell residue. The centrifuging process was repeated, usually three times, until no visible detritus was thrown down. With sufficient care in removing the supernatant after each centrifugation, extracts could be obtained which were sterile, as judged by sterility tests in blood media. However, to remove any possible doubt as to the presence of living cells the extracts were filtered through Berkefeld candles.

The sterility of the filtered extracts was always controlled by plating 0.4 cc. in blood agar and by adding 0.4 cc. to blood broth. The filtered extracts were stored in the ice box in long, narrow tubes under vaseline seal.

These extracts reacted with the oxygen of the air with such avidity that preparations which were peroxide-free before filtration, gave strong peroxide tests immediately after the filtration process. Even if cooled to 2°C., the extracts could not be kept peroxide-free, although the whole filtration apparatus was packed in ice. However, the amount of peroxide formed during filtration was rapidly decomposed when the filtered extract was subsequently sealed with vaseline to prevent further oxidation. The extract rendered peroxide-free by its own reducing action under anaerobic conditions, was again able to form peroxide when exposed to the action of molecular oxygen.

Since the oxidizing-reducing activity of the extract might be impaired by even the transient presence of small amounts of peroxide, it seemed desirable to reduce to a minimum the contact of the extract with oxygen during filtration. In later preparations, the filtration procedure was modified to meet this end.

Extracts were cooled to 2°C., the entire filtration apparatus was packed in ice, and the filtrate was collected in a long narrow tube instead of the usual flasks. With these precautions, the clear extracts could be filtered with minimum aeration at low temperature without the formation of significant amounts of peroxide.

To prevent contact of the extract with oxygen during filtration, an apparatus was devised which permitted the preparation to be filtered and collected in an atmosphere of pure nitrogen. The latter filtration procedure was employed in the preparation of all extracts used in the experiments reported in following papers of this series.

Peroxide Formation by Sterile Cell Extracts.—Sterile preparations of unwashed pneumococci, extracted in broth or phosphate solution in the manner described, contain a complete peroxide-forming system as shown by their ability to unite with or "accept" oxygen from the air with the prompt formation of peroxide. The sterile broth extracts, since they possessed greater activity than the extracts prepared in phosphate solution, have been used in the following experiments. The most active extracts are those which have been subjected to the minimum exposure to oxygen throughout their preparation.

Extracts which are exposed to the air for short periods and in which peroxide has been formed, may be rendered peroxide-free by the reducing action of the extract itself under anaerobic conditions. Such extracts, now peroxide-free, still possess the property of forming peroxide when again exposed to air. However, if exposure to oxygen, and to peroxide, is prolonged, the extract, after anaerobic destruction of the peroxide, shows a gradual diminution and final total loss of its former power of uniting with oxygen to form peroxide. The possible deleterious action of peroxide itself upon the activity of the system

complicates an analysis of the above phenomenon in terms of reversibility of oxidation and reduction. Experiments are planned to furnish data on this question.

Influence of Temperature.—The reduced extract shaken in the air at 20°C. for a few minutes gives a strong peroxide test. Peroxide is formed almost as readily when the extract is aerated at 2°C. The promptness with which peroxide formation takes place at these temperatures is indicative of the avidity with which the reduced extract unites with molecular oxygen.

Influence of H Ion Concentration upon Peroxide Formation by Sterile Extracts of Pneumococcus.

The following experiment was conducted to determine the influence of pH upon peroxide formation by the sterile extracts of the intracellular substances of pneumococcus.

TABLE I.

Influence of pH upon Peroxide Formation by Sterile, Cell Extracts of Pneumococcus.

pH at which extract was exposed to air.	Peroxide tests after aerobic exposure at 20°C. for 1 hr.
4.0	±
5.0	++
6.0	+++
7.0	+++
8.0	+++
9.0	+++

In Tables I to III − indicates that no peroxide was detected; ±, faint reaction; +, weak reaction; ++, moderate reaction; +++, marked reaction; and ++++, strong reaction.

0.5 cc. of sterile extract was mixed in small tubes with 1 cc. of sterile glycine-phosphate-acetate buffer solutions (2) at different pH. The tubes were sealed with vaseline and incubated at 20°C. for 1 hour as a further insurance of the absolute absence of peroxide in the adjusted extract before its exposure to air. The mixture was then transferred to another tube and shaken in the air. Aeration was continued by placing the tubes in a horizontal position in the dark.

At the intervals shown in Table I peroxide tests were made. Control tests of the extract at each pH were peroxide-free before exposure to the air.

Table I shows that detectable amounts of peroxide may be produced by the extracts within a reaction range of pH 5.0 to 9.0. However, as shown by the intensity of the reaction, the most active peroxide formation occurred in a zone between pH 6.0 and 9.0.

Influence of Heat upon the Peroxide-Forming Power of Sterile, Broth Extracts of Pneumococcus.

Influence of Heating at 55°C.—The following experiment is concerned with (1) the stability of the peroxide-forming system of cell extracts at 55°C., and (2) a comparison of the influence of heat upon the peroxide-forming ability of sterile extracts with that of the intact cell itself.

TABLE II.

Effect of Heating at 55°C. upon the Peroxide-Forming Activity of Sterile, Cell Extracts and of Suspensions of Whole Cells.

Extract or suspension heated at 55°C. for.	Peroxide-forming activity.	
	Filtered sterile cell extract.	Suspension of whole cells.
min.		
10	+++	++
20	+++	−
30	++	−
40	+	−
50	+	−
60	±	−
Unheated control.	++++	++++

Cell Suspensions.—A 12 hour anaerobic broth culture of Pneumococcus Type II (Strain G) was centrifuged and the cells were taken up in the supernatant so that 1 cc. of the fluid suspension contained the bacterial cells from 25 cc. of culture.

Extract.—The sterile extract used in this experiment was prepared from 35 cc. of anaerobic broth culture of Pneumococcus Type II (Strain G).

Heating.—1.5 cc. of extract, or of cell suspension, were placed in a series of small agglutination tubes, sealed with vaseline, and incubated 20 minutes to insure anaerobic conditions during heating. One tube of each preparation was then immersed in a constantly stirred water bath at 55°C. for the period noted. Each tube was cooled immediately after heating and left standing under seal in the water bath at 15°C. until all tubes of the series had been heated. The contents of each tube were then transferred to another tube, shaken, and exposed to the air in the dark at 20°C.

Peroxide tests were made after 1 hour's aeration. Control tests proved the absence of peroxide in all cases before exposure to the air. Results are given in Table II.

As shown in Table II, the peroxide-forming activity of pneumococcus extract gradually diminished on heating at 55°C. However, after exposure of 1 hour at 55°C., the extract was still able to form small amounts of peroxide. On the other hand, suspensions of intact cells heated for 20 minutes or longer, lost all power of forming peroxide when exposed to air. This relation is especially interesting since in both instances the same strain of pneumococcus was used and the concentration of cells, from which the extract and the suspension had been prepared, was comparable. In the case of the suspension of bacteria the altered permeability of the cell membrane due to the action of heat may prevent the functioning of the peroxide-forming system even though the potential ability of cellular constituents to unite with oxygen and form peroxide has not been utterly destroyed.

Effect of Heating at Different Temperatures.—The preceding experiment has shown that continued exposure to 55°C. gradually destroys the peroxide-forming activity of sterile, pneumococcus extract. To obtain further information on the degree of thermostability of the peroxide-forming system, sterile extracts were exposed for a period of 5 minutes to temperatures ranging from 55° to 100°C.

1.5 cc. of cell extract were placed in narrow agglutination tubes, sealed with vaseline, and incubated 30 minutes at 37°C. The series of tubes was then immersed for exactly 5 minutes in a constantly agitated water bath at the temperatures shown in Table III. The tubes were cooled immediately in ice water and held under seal until all heating tests were completed.

The heated extract in each instance was then placed in tubes, shaken, and exposed to the air in a thin layer at room temperature. Samples were taken from these tubes for the peroxide tests. Results are given in Table III.

Table III reveals the fact that 5 minutes exposure to 55°C. has but slight effect upon the peroxide-forming activity of the extract. Indeed, as shown in the preceding experiments, these extracts may be subjected for an hour to 55°C. without complete loss of this function. 5 minutes exposure at 65°C., however, resulted in the

complete destruction of the system responsible for peroxide production. Subsequent experiments have shown that these extracts after 10 minutes exposure to 60°C., show marked ability to form peroxide. The critical temperature for the destruction of this activity, therefore, would seem to lie between 60° and 65°C.

TABLE III.

Effect of Heating at Different Temperatures upon Peroxide Formation by Sterile Extracts of Pneumococcus.

Cell extract heated for.	Peroxide formation after exposure to air for 1 hr.
5 min. at 55°C.	+++
5 " " 65° "	−
5 " " 75° "	−
5 " " 85° "	−
5 " " 100° "	−
Unheated control.	++++

Activation of Saline Extracts of Washed Pneumococci.

In the preceding experiments, broth and saline extracts of unwashed pneumococci have been shown to possess an active peroxide-forming system. In the following experiments, an attempt has been made to determine whether extracts possessing this same property could be prepared by the extraction of washed bacteria in salt or phosphate solution.

The technique employed in the preparation of saline extracts of washed cells was the same as that used in preparing broth extracts of unwashed pneumococci, with the following exception: the centrifuged cells instead of being extracted directly were washed several times in sterile water or salt solution and finally extracted in 0.05 M sterile phosphate solution, pH 7.5.

0.5 cc. of unfiltered sterile extract of washed pneumococci was added to an equal volume of meat infusion[1] or yeast extract[1] which had been adjusted previously

[1] Muscle infusion: The infusion was prepared by the routine procedure followed in the laboratory in the preparation of meat infusion broth. 500 gm. of lean beef were allowed to infuse in 1 liter of distilled water overnight at ice box temperature. After filtration through coarse paper, the infusion was sterilized for 20 minutes at 100°C. on 3 successive days. The reaction was adjusted to pH 7.5 before use.

Yeast extract: 100 gm. of brewer's yeast in 400 cc. of distilled water were adjusted to pH 4.5 and boiled for 10 minutes. The cellular material was allowed

to pH 7.5. A double quantity of washed cell extract alone served as control. As further controls 0.5 cc. amounts of yeast extract or meat infusion without pneumococcus extract were added to 0.5 cc. of sterile phosphate solution. All of these mixtures were then aerated at 20°C. for 1 hour and then tested for the presence of peroxide. The results are recorded in Table IV.

The differences between the two types of extracts consist in the kind of medium used for extraction and in the removal of certain substances during the process of cell washing. Since sterile broth extracts of unwashed pneumococci form peroxide actively on exposure to molecular oxygen, they may be said to possess a complete peroxide-forming system which requires only the presence of molecular oxygen

TABLE IV.

Activation of Peroxide-Forming Power of Sterile Extracts of Washed Pneumococci.

Sterile extract of washed pneumococci.	Activating substance.		Phosphate solution pH 7.5.	Peroxide formation after 1 hr.'s aeration at 20°C.
	Meat infusion.	Yeast extract.		
cc.	*cc.*	*cc.*	*cc.*	
1.0				−
0.5	0.5			+
0.5		0.5		+
	0.5		0.5	−
		0.5	0.5	−

In this table − indicates negative peroxide reaction; +, positive peroxide reaction.

to initiate the process of peroxide formation. The saline extracts of washed bacteria, on the other hand, are by themselves unable to form peroxide when exposed to air (Table IV). The fact, however, that the addition of certain substances to these washed cell extracts serves to activate them in the presence of oxygen, indicates that they contain an incomplete peroxide-forming system. The behavior of these two types of cell extract is wholly analogous to that of the corresponding suspensions of living bacteria from which the extracts are derived. Suspensions of unwashed pneumococci in broth possess in common with the sterile broth extracts of unwashed cells this

to sediment at room temperature and the clear supernatant was pipetted off and tested for sterility. The extract was stored in the ice box and the reaction adjusted to pH 7.5 before use.

peroxide-forming function; similarly, saline suspensions of washed pneumococci, like the sterile saline extract of washed cells, lack this function unless reactivated by the addition of some accessory substance or substances.

Several substances have been found to serve as activators of peroxide-forming activity in cell suspensions and in sterile extracts in which this function has ceased. As might be expected, extracts of washed cells regain the property of forming peroxide by the simple addition of the cell washings. Moreover, sterilized broth such as is used for bacteriological purposes, heated meat infusion, and boiled yeast extract are also able to complement the otherwise deficient peroxide-forming system in saline extracts of washed pneumococci.

The nature of the activating substances in cell washings, meat infusion, and yeast extract is not wholly clear. However, the analogy between the completion of the peroxide-forming system of washed pneumococci and the stimulation by similar substances of the respiratory activity of washed tissue, suggests that here too the principal rôle of these activators may be the furnishing of easily oxidized, or autoxidizable substances.

SUMMARY.

In the present paper methods have been described for the preparation of sterile extracts of pneumococci. These extracts may be obtained by dissolving the bacteria in broth cultures by means of bile, or by extraction of the cellular substances by repeated freezing and thawing of broth or saline suspensions of unwashed cells. Under special precautions these extracts may be passed through Berkefeld filters without loss of potency. In this procedure, as in all other manipulations incident to their preparation, the extracts should be protected as far as possible from contact with air. All extracts were proved sterile by cultural and animal tests.

Sterile extracts of unwashed pneumococcus cells promptly form peroxide on exposure to air. Peroxide formation is almost as active in extracts aerated at 2°C. as in those exposed to the air at room temperature. Detectable amounts of peroxide may be produced by these cell extracts within the reaction range of pH 5 to 9, the optimal zone lying at reactions less acid than pH 6. The peroxide-

forming activity of the extracts is gradually diminished by prolonged exposure to 55°C., and is completely destroyed by heating at 65°C. for 5 minutes.

Cell extracts of pneumococci which have been thoroughly washed prior to extraction in salt or phosphate solutions exhibit no peroxide-forming activity. These extracts of washed cells may be activated by the addition of the cell washings, yeast extract, or muscle infusion.

CONCLUSIONS.

The peroxide-forming activity of pneumococcus is a function not dependent upon the presence of living cells.

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QUANTITATIVE FACTORS IN "TEST-TUBE INFECTION."

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One of the most important problems in epidemiology is the analysis of that train of circumstances which leads to the spread of infection in extensive or epidemic form. In the past, the question has been attacked largely by reflective methods, and one finds that stress has been placed on the influence of weather, of season, of sudden accessions of virulence of bacteria, or that the difficulty has been evaded by deferring to the "genius" of the epidemic, but none of these hypotheses afford scientifically acceptable explanations. Consideration of healthy carriers of pathogenic bacteria, which has been the key-note of epidemiological study in recent years, seemed at first to offer a solution for the obscure phenomena of the spread of infectious disease, but important as the principle of the carrier state may be, and while it makes clear the location of storehouses of bacteria which may later give rise to clinical disease, it fails to indicate why carriers of virulent diphtheria bacilli, meningococci, streptococci, and other germs dwell harmless in our midst for long periods of time only to lead to epidemics later in ways that are unexplained. It is now generally recognized that accurate simultaneous analyses of many factors both clinical and bacteriological are necessary if advances in knowledge are to be made. An expression of this feeling is found in the recent work of Topley (1), of Amoss (2), and of Webster (3) on animal epidemics which were propagated under rigidly controlled conditions.

But despite the obscurity which involves the mode of genesis of epidemic disease a few facts stand out clearly defined. There can be no doubt that crowding, especially when large aggregations of people are assembled in close and intimate contact, promotes the

spread of infection. This crude principle obviously of elementary importance demands more detailed analysis and if possible presentation in something approximating mathematical form.

The experiments which will be described were suggested by certain observations made in connection with an outbreak of acute tonsillitis among the students of the Johns Hopkins Hospital Training School for Nurses (4). During the winter of 1922–23 there occurred in this group of about 200 young women some forty cases of tonsillitis. Inasmuch as there were many chronic carriers of hemolytic streptococci in the group it was assumed at first that the disease was acquired by susceptibles from carriers during the course of the general contact which existed in the wards of the hospital, in the dining room, assembly, etc., but when the strains recovered from various cases were identified it was found that in nearly every instance one was dealing with dormitory infection. This observation seemed to indicate that a high degree of intimate contact was necessary before clinical infection came about, although there was undoubtedly real opportunity for the susceptibles to acquire hemolytic streptococci elsewhere than in the living quarters (5).

It was necessary then to consider the mechanism of respiratory infection from a different point of view. The observations discussed above suggested that in the case of a population whose resistance to a certain organism is in general high a certain critical dose, other things being equal, must be acquired before disease will be produced, and that even in the presence of many sources of infection and even if the causal organisms actually enter the upper air passages, any contact which leads to the acquisition of bacteria in an amount smaller than such a critical dose will be inadequate to produce infection at all. If this hypothesis is correct the perplexing problem of why the chronic carrier of pathogenic bacteria may exist in a community for long periods of time without consequent infection of susceptibles is immediately explained. The following experiments represent an attempt to reproduce in the test-tube conditions analogous to those of human infection and to control them with a certain degree of mathematical accuracy.

General Methods.

Throughout this work a strain of beta-hemolytic streptococcus was used. It had been isolated originally from a case of acute tonsillitis and was shown to be colony-pure by frequent platings. The organism was selected because it was easy to prepare media which were either favorable or antagonistic to its growth. Test-tubes containing accurately measured amounts of media of various kinds were used to represent susceptible or relatively insusceptible people or animals. The criterion of "infection"[1] was taken to be the multiplication of the bacteria in the test medium as demonstrated by macroscopic growth and by colony counts in plated samples. The total volume of inoculum for all tubes was constant in each experiment (0.5 cc. of various dilutions of the culture) and the number of viable organisms in the inoculum was always controlled by preliminary plating in a favorable medium (ascites-meat infusion agar pH 7.4). The counting of streptococci is to some extent inaccurate since a colony on the plate indicates a chain and not a single organism but as the growth of the strain was uniform and very diffuse the results were regarded as comparable. Platings were made on blood agar at the end of the experiments to be sure that no contamination had been introduced.

It may be noted that the number of organisms demonstrated by preliminary control platings did not always vary directly with the amount of culture. This is due to the adherence of bacteria to the walls of the pipette in the higher dilutions but is of no importance in the interpretation of the experiments.

Relation of Dosage of Bacteria to Test-Tube Infection.

The first set of experiments was devised to discover whether in dealing with inoculation into relatively unfavorable media there was a critical dose which would produce infection in 100 per cent of cases, and whether any material lessening of the dose would lead not to a relatively smaller percentage of infections but to a number approximating zero.

Experiment 1.—Strain 205, beta-hemolytic streptococcus, originally isolated from a case of acute tonsillitis; has been kept in stock for about 4 months. 48 hour culture in meat infusion broth pH 7.4 inoculated into sets of four tubes each containing 5 cc. of the same medium. Increasing dilutions of the culture, made up in each case to a total volume of 0.5 cc., were inoculated into successive sets of tubes.

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[1] Throughout this paper no attempt is made to use the terms "infection," "virulence," "susceptibility," etc., in an accurate technical sense except when specially indicated. The implication is clear from the context.

Amount of culture.	Control plating (No. of colonies).*	24 hr. readings.			
		Tube 1.	Tube 2.	Tube 3.	Tube 4.
cc.					
1/500		+†	+	+	+
1/1,000		+	+	+	+
1/2,000		+	+	+	+
1/4,000		+	+	+	+
1/8,000	1,200	+	+	+	+
1/16,000	700	+	+	+	+
1/32,000	400	+	+	+	+
1/64,000	350	+	+	+	+
1/12,800	230	+	+	+	+
1/25,600	250	+	+	+	+
1/51,200	150	+	+	+	+
1/102,400	110	+	+	+	+
1/204,800	65	+	+	+	+

* Number of viable bacteria in the inoculum.

† In the tables + indicates infection (macroscopic growth with innumerable colonies on plating).

Experiment 2.—Strain 205, beta-hemolytic streptococcus. 4 day culture in meat infusion broth pH 7.4 inoculated in increasing dilutions into sets of three tubes of 5 cc. of same medium. Same technique as in Experiment 1.

Amount of culture.	Control plating (No. of colonies).	24 hr. readings.		
		Tube 1.	Tube 2.	Tube 3.
cc.				
1/4		+	+	+
1/16		+	+	+
1/32		+	+	+
1/64		+	+	+
1/128		+	+	+
1/256		+	+	+
1/500		+	+	+
1/1,000		+	+	+
1/2,000		+	+	+
1/4,000	600	+	+	+
1/8,000	300	+	+	+
1/16,000	175	+	+	+
1/32,000	71	+	+	+
1/64,000	26	+	+	+

Experiment 3.—Strain 205, beta-hemolytic streptococcus. 4 hour culture in meat infusion broth pH 7.4 inoculated in increasing dilutions into sets of two tubes of 5 cc. of same medium. Same technique as in Experiment 1.

Amount of culture.	Control plating (No. of colonies).	24–48 hr. readings.	
		Tube 1.	Tube 2.
cc.			
1/8		+	+
1/16		+	+
1/32		+	+
1/64		+	+
1/128	15,000	+	+
1/256	5,000	+	+
1/500	350	+	+
1/1,000	2	+	+
1/2,000	2	+	+
1/4,000	1	+	+
1/8,000	0	+	+
1/16,000	1	+	+
1/64,000	0	+	+
1/128,000	0	+	+

This preliminary group of experiments was carried out to determine the ease of establishment of growth of the test organism in a highly favorable medium (meat infusion broth pH 7.4). It is seen that within 24 to 48 hours profuse macroscopic growth occurred uniformly in all sets of tubes although the inoculum in some was so small that control plating yielded from 0 to 2 colonies. This state of affairs may be thought of as analogous to the presence of a source of infection among a uniformly and extremely susceptible population. Under these conditions it is known that disease may occur in practically 100 per cent of those who presumably acquire even a minute dose of the virus.

The next set of experiments was designed to represent the exposure of sets of relatively resistant individuals to decreasing amounts of the same organisms used in Experiments 1 to 3. For this purpose tubes of meat extract broth pH 7.4, a medium unfavorable to the growth of hemolytic streptococci, were used instead of tubes of meat infusion broth. Otherwise conditions were unchanged.

Experiment 4.—Strain 205, beta-hemolytic streptococcus. 19 hour culture in meat infusion broth pH 7.4 inoculated in increasing dilutions into sets of five tubes of 5 cc. of meat extract broth pH 7.4. Same technique as in Experiment 1.

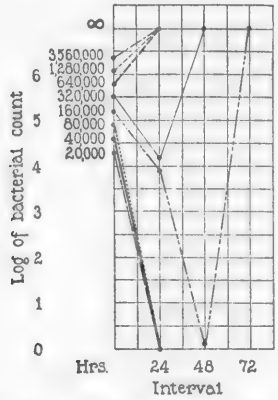
Amount of culture.	Control plating (No. of colonies).	24 hr. readings.					Plating of Tube 5 of each set after 24 hrs. (No. of colonies per tube).
		Tube 1.	Tube 2.	Tube 3.	Tube 4.	Tube 5.	
cc.							
1/4	3,560,000	+	+	+	+	+	∞*
1/8	1,280,000	+	+	+	+	+	∞
1/16	640,000	+	+	+	+	+	∞
1/32	320,000	0	0	0	0	0	16,000
1/64	160,000	0	0	0	0	0	8,000
1/128	80,000	0	0	0	0	0	0
1/256	40,000†	0	0	0	0	0	0
1/500	20,000†	0	0	0	0	0	0
1/1,000	10,000‡	0	0	0	0	0	0
1/4,000	2,500‡	0	0	0	0	0	0

* ∞ indicates innumerable colonies.

† Estimated.

‡ Counted.

The protocol shows that while multiplication took place in 100 per cent of the tubes which received 640,000 bacteria as evidenced by macroscopic growth in 24 hours, none of the tubes which received 320,000 bacteria were infected. Furthermore, plating at the end of



TEXT-FIG 1. Graph of Experiment 4.

24 hours indicated that in the tubes which showed no macroscopic growth the number of viable organisms had decreased tremendously. The significant point then is that one-half of the dose which produced infection in 100 per cent of the series produced infection not in 50 per cent but in zero per cent; any dose short of the critical one was inadequate in spite of the large number of viable organisms which had been introduced as determined by preliminary plating.

The subsequent events in this experiment as revealed by daily macroscopic inspection and by plating were of great interest. They will be discussed later in connection with lag. It is seen from Text-fig. 1 that the larger inocula multiplied rapidly, reaching ∞ (macroscopic growth with innumerable colonies) in 24 hours. At 320,000 bacteria, however, there was a decided difference—multiplication did not occur; on the contrary at 24 hours the count in this tube was 16,000 and in the succeeding tube 8,000. All smaller inocula gave zero counts at 24 hours and there never was any growth in these tubes. At 24 hours another marked change is seen; the fourth tube now containing 16,000 bacteria showed rapid growth which reached ∞ at 48 hours, but in the fifth tube there was a further drop in the count to only 5 colonies. But now there occurred in this tube also a rapid growth so that at 72 hours it reached ∞.

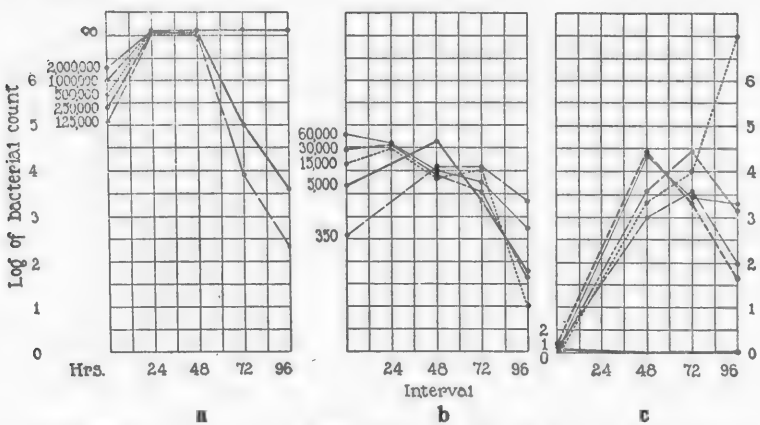
The next experiment was similar to Experiment 4.

Experiment 5.—Strain 205, beta-hemolytic streptococcus. 4 hour culture in meat infusion broth pH 7.4, diluted in salt solution and inoculated into meat extract broth pH 7.4 as in Experiment 4.

Amount of culture.	Control plating (No. of colonies).	24 hr. readings.			Plating of Tube 3 of each set after 24 hrs. (No. of colonies per tube).
		Tube 1.	Tube 2.	Tube 3.	
cc.					
1/2	2,000,000	+	+	+	∞
1/4	1,000,000	+	+	+	∞
1/8	500,000	+	+	+	∞
1/16	250,000	+	+	+	∞
1/32	125,000*	+	+	+	∞
1/64	64,000†	0	0	0	40,000
1/128	32,000†	0	0	0	40,000
1/256	15,000	0	0	0	35,000
1/500	5,000	0	0	0	
1/1,000	350	0	0	0	
1/2,000	2	0	0	0	
1/4,000	2	0	0	0	
1/8,000	1	0	0	0	
1/ 16,000	0	0	0	0	
1/ 32,000	1	0	0	0	
1/ 64,000	0	0	0	0	
1/128,000	0	0	0	0	

* Estimated.

† Counted.



TEXT-FIG. 2, *a* to *c*. Graph of Experiment 5. The three parts of the diagram are charted separately to avoid crowding of the curves and should be regarded as superimposed.

The result of this experiment is similar to that of Experiment 4. There was a critical dose which infected 100 per cent of each set of tubes, the dose immediately succeeding infected none of the next set of tubes. Subsequent events in this experiment are indicated in Text-fig. 2. It is seen that doses of 125,000 or more bacteria multiplied rapidly and reached ∞ in 24 hours. There is again a critical change at this point and in the next few tubes there occurred what might be regarded as analogous to a temporary carrier state, the counts were practically unchanged at 24 hours. The third group of inocula of only a very few organisms eventually led to marked multiplication as shown in Text-fig. 2, *c*, but except in the case of one tube never went ahead to ∞. This finding will be discussed at a later point in this paper.

The next experiment was similar to the two preceding except that an attempt was made to attain greater refinement by decreasing the dose in successive sets of tubes by one-fourth instead of by one-half.

Experiment 6.—Strain 205, beta-hemolytic streptococcus. 24 hour culture in meat infusion broth pH 7.4 inoculated in decreasing dilution into sets of five tubes of 5 cc. of meat extract broth pH 7.4.

Amount of culture.	Control plating (No. of colonies).	24 hr. readings. Tube No.					48 hr. readings. Tube No.					Plating after 48 hrs. (No. of colonies per tube).
		1	2	3	4	5	1	2	3	4	5	
cc.												
1/3		+	+	+	+	+	+	+	+	+	+	∞
1/4		+	+	+	+	+	+	+	+	+	+	∞
1/6		+	+	+	+	+	+	+	+	+	+	∞
1/8	1,280,000	+	+	+	+	+	+	+	+	+	+	∞
1/12	960,000	+	+	+	+	+	+	+	+	+	+	∞
1/16	640,000	+	+	+	+	+	+	+	+	+	+	∞
1/24	480,000	+	+	+	+	+	+	+	+	+	+	∞
1/32	320,000	0	0	0	0	0	0	0	0	0	0	55,000
1/48	240,000	0	0	0	0	0	0	0	0	0	0	23,000
1/64	160,000	0	0	0	0	0	0	0	0	0	0	22,500
1/96	120,000	0	0	0	0	0	0	0	0	0	0	15,000
1/128	80,000	0	0	0	0	0	0	0	0	0	0	18,000
1/192	60,000	0	0	0	0	0	0	0	0	0	0	3,000
1/256	40,000	0	0	0	0	0	0	0	0	0	0	5,000

In this experiment as in the preceding ones a critical point occurred below which no infection took place in spite of the relatively small difference between successive doses.

Many other similar experiments were done which all yielded the same result. In some cases the bacteria were centrifuged and resuspended before inoculation in order to rule out any confusing effect which might be due to varying amounts of the original culture material. Such a procedure in no way altered the phenomenon which has been described.

The essential feature of the preceding experiments has been the demonstration that when bacteria are placed in a relatively unfavorable medium in decreasing amounts a critical point is reached at which growth is uniformly inhibited. The point especially to be emphasized is that any dose materially short ot that capable of producing active growth in 100 per cent of a set of tubes leads to growth not in a proportionally smaller number of tubes but in none of them despite the introduction of large numbers of viable organisms. While the dose decreases arithmetically the percentage of infections falls abruptly to zero.

In dealing with the practical problem of the control of infectious disease the question of contact has caused a good deal of confusion. From the technical public health standpoint the presence of a susceptible individual in the vicinity of a source of infection so that contagion theoretically might have been acquired is regarded as contact, but as is well known clinical infection often fails to follow. In fact carriers of pathogenic bacteria such as the diphtheria bacillus or hemolytic streptococcus may live in considerable intimacy with susceptibles without the spread of disease or the development of other carriers. It is unlikely that these people who are exposed never actually acquire organisms from the carrier. There is some factor which prevents infection except under special circumstances. If the phenomenon which has been demonstrated in the test-tube held true *in vivo* the matter would be to some extent explained. We shall consider therefore several carefully controlled studies of human epidemics in this light.

Epidemic Meningitis.—The well known observations of Glover at the Caterham Depot (6) are especially significant. Among a military unit quartered in

roomy barracks the meningococcus carrier rate was repeatedly found to be from 0.5 to 4 per cent, an essentially normal figure. When later, owing to the exigencies of the war, it was necessary to overcrowd the quarters and to reduce the spacing of the beds from 3 feet to 1 foot or less the rate immediately began to rise, reaching even the amazing figure of 70 per cent coincidently with an outbreak of clinical meningitis. As long as the close spacing of the beds was maintained the carrier rate continued excessively high, but as soon as the distance between beds was again increased to 2½ feet the rate fell, gradually reaching normal, and meningitis no longer occurred. This sequence of events was twice repeated, indicating beyond doubt that the close approximation of the men in the sleeping quarters was the factor which lead to spread of meningococcus infection.

Diphtheria and Scarlet Fever.—Dudley (7) reports carefully controlled studies of an outbreak of diphtheria and of scarlet fever in a boys' school. It was noteworthy that all the cases occurred among the students who lived in the school and slept in the dormitories although there was also a group of day boys who mingled freely with the others " . . . . Over 100 boys (day students) played, worked, and ate for over a year without returning a single case of diphtheria or scarlet fever, while 300 of the boys with whom they played, worked, and ate (resident students) developed one or the other of these diseases."

Influenza.—During the influenza epidemic of 1918 nearly 40 per cent of the nursing staff of the Johns Hopkins Hospital developed influenza (8). At the same time only six of the patients in bed on the general wards were affected although they were attended by a staff which was constantly being stricken by the disease. The relative immunity of the bed patients cannot be explained by total lack of exposure but must have been due to a lower degree of contact than existed among the ambulatory group of attendants.

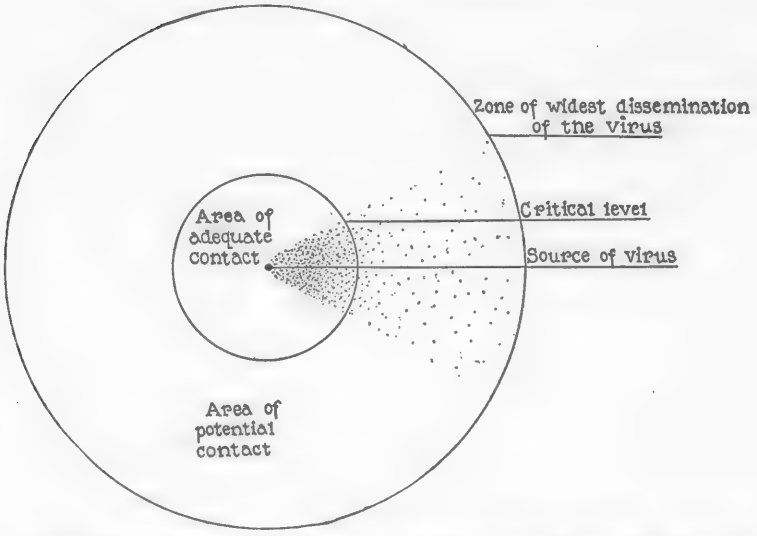
Tonsillitis.—Bloomfield and Felty (5) in a study of an outbreak of tonsillitis in a limited group of people showed that while healthy carriers of streptococcus were the sources of infection, specially close and intimate contact was necessary before clinical infection came about. General contact among the members of the group seemed to lead not to a proportionately smaller number of infections but apparently was inadequate.

A consideration of these examples reveals a state of affairs strikingly analogous to that which holds in the test-tube experiments. In each instance the one variable upon which seemed to depend the occurrence or non-occurrence of infection was the degree and constancy of proximity of the individual to the source of the causal organisms; *i.e.*, the size of the dose. Studies of droplet distribution indicate that infected particles may be sprayed for a considerable distance, as far as 30 to 40 feet. It is clear, therefore, that many of the susceptibles in the examples quoted above were actually receiving pathogenic bacteria

even at a time when no clinical infection occurred; it was only when crowding or contact reached some critical point that there was an abrupt transition from almost complete absence of infection to a high incidence of disease, and *vice versa*, below the critical point infection was not less in arithmetical proportion but became practically zero.

It seems then that in the case of a population which is relatively resistant to a certain type of infection one must consider contact of two sorts. First, one may speak of potential contact, which simply indicates that the individual has been within the widest possible range of distribution of the virus from its source. Secondly, one has to deal with adequate contact, which may actually be followed by clinical infection and which comes about only when the person has been in such proximity to the source of the virus that a dose greater than some critical value may be acquired. As has been indicated it seems that the likelihood of infection does not vary directly with the degree of exposure but drops suddenly at a certain point from a relatively high figure to approximately zero. As a concrete example one may consider a diphtheria carrier and his associates. The carrier constantly or from time to time distributes diphtheria bacilli to the outside world and these organisms are doubtless at times acquired by his associates. The number of organisms acquired, however, during such association as ordinary conversation or presence in the same room may be too few to produce infection (potential contact), whereas if the carrier coughs frequently and directly into the face of the associate the transferred dose may surpass the critical point and infection results (adequate contact). Guthrie (9) and his collaborators followed a large number of diphtheria carriers for over a year. During this time no case or secondary carrier appeared among the contacts. But the cultures (large dose) from the carriers sprayed into the throats of volunteers produced diphtheria almost uniformly.

In summary then, it may be that the usual harmlessness of respiratory tract carriers of pathogenic bacteria is due to the fact that the contacts acquire only inadequate doses of the organisms under ordinary conditions, and that an unusual and high degree of association is necessary, other things being equal, before an adequate dose is transferred. The situation may be diagrammed as shown in Text-fig. 3.


Zone of widest dissemination of the virus
Critical level
Source of virus
Area of adequate contact
Area of potential contact

TEXT-FIG. 3. Diagrammatic representation of potential contact and adequate contact.

Summation of Dosage.

In the previous section it has been pointed out that in the case of an unfavorable medium a relatively large dose of bacteria is necessary to produce growth. The question naturally follows whether under these conditions there is some true synergistic action of bacteria which only comes about when a certain number are brought together in the medium or whether a dose of a certain magnitude is necessary before there will be included in it enough organisms in such a state of activity as to be able to multiply.

As far as the test-tube is concerned true synergistic or communal activity seems to have been demonstrated.

Churchman (10) working with single bacterial cells showed that under the conditions of his experiments a minimum inoculation of thirty organisms was necessary to produce growth in 5 cc. of medium; smaller numbers were inadequate. On the other hand, thirty organisms would also produce growth in a much larger quantity of medium, as much as 1,000 cc. This phenomenon Churchman refers to as the communal activity of bacteria. One may mention also the observation

of Gillespie (11) that multiplication of pneumococci in broth may be more readily initiated if the bacteria are floated on a bit of filter paper. Inasmuch as in natural contact the entire dose of virus is not always received at once but may be acquired in successive fractions at various time intervals it seems of the utmost importance to determine whether the consummated infection results from true synergistic action of multiple doses or whether such multiple doses merely afford more opportunity for a vegetative organism to be deposited at a favorable site for growth.

Dudley (7) has recently discussed this matter from the clinical standpoint and has brought out a principle which he refers to as the "velocity of infection." Its essential feature is the conception that the consummated infection comes about only when the virus attains a certain concentration in the susceptible, and that the critical dose may be the resultant of an accumulation of successively received subinfective doses.

"For the sake of argument, suppose that all the conditions are fixed and constant. An individual receives V amount of infective agent per hour. The defensive mechanism can destroy U amount of this infective agent per hour; then we may term V the velocity at which infection is received and U the velocity of destruction of infection. If U is greater than V, it follows that no infection can ever take place so long as the conditions remain unchanged, because the infective agent is being dealt with as fast as it can be received. But if V is greater than U, infection can take place, but only if conditions are in operation long enough. The time required will depend on how much greater V is than U, that is, on $V - U$, which is the amount of the infective agent left undestroyed at the end of each hour, and therefore $V - U$ is the velocity at which infection takes place. The nearer U (the velocity of destruction) approaches V (the velocity of reception), the smaller $V - U$ (the velocity of infection) becomes. . . . . V will of course vary with the source of infection and its distance, and with the conditions of ventilation and humidity. It is now easy to see that for a subject to collect a M. I. D. (minimum infecting dose) of an infective agent he must remain $\frac{\text{M. I. D.}}{V - U}$ hours in the same infective environment."

While Dudley's idea seems to harmonize with many observed facts it is none the less hypothetical, and experimental demonstration of the principle would be of interest.

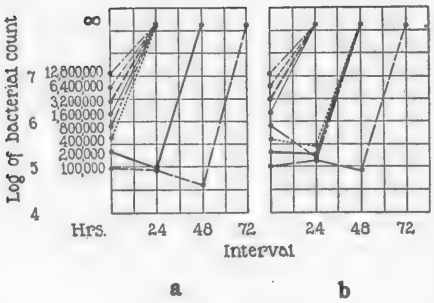
The next experiments deal, therefore, with the question of communal activity of bacteria and effect of summation of dosage from the test-tube standpoint.

Experiment 7. Relation of Dose and Volume of Medium to Growth.—Strain 205, 28 hour culture. Centrifuged and resuspended in salt solution. Decreasing amounts inoculated into sets of four tubes of meat extract broth, two of 5 cc. and two of 10 cc.

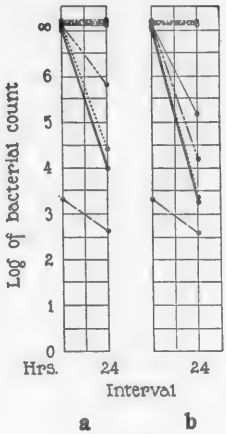
Amount of culture.	Control plating (No. of colonies).	24 hr. readings. Tube No. 10 cc. 1	10 cc. 2	5 cc. 3	5 cc. 4	Plating after 24 hrs. Tube No. 1	Tube No. 3	48 hr. readings. Tube No. 1	2	3	4	Plating after 48 hrs. Tube No. 1	Tube No. 3
cc.													
1/2	12,800,000	+	+	+	+	∞	∞	+	+	+	+	∞	∞
1/4	6,400,000	+	+	+	+	∞	∞	+	+	+	+	∞	∞
1/8	3,200,000	0	0	+	+	∞	∞	+	+	+	+	∞	∞
1/16	1,600,000	0	0	+	+	∞	∞	+	+	+	+	∞	∞
1/32	800,000	0	0	+	+	168,000	∞	+	+	+	+	∞	∞
1/64	400,000	0	0	+	+	240,000	∞	+	+	+	+	∞	∞
1/128	200,000	0	0	0	0	180,000	90,000	+	+	+	+	∞	∞
1/256	100,000	0	0	0	0	180,000	84,000	0	0	0	0	96,000	42,000

Amount of culture.	72 hr. readings. Tube No. 1	2	3	4	Plating after 72 hrs. Tube No. 1	Tube No. 3	96 hr. readings. Tube No. 1	2	3	4
cc.										
1/2	+	+	+	+	∞	∞	+	+	+	+
1/4	+	+	+	+	∞	∞	+	+	+	+
1/8	+	+	+	+	∞	∞	+	+	+	+
1/16	+	+	+	+	∞	∞	+	+	+	+
1/32	+	+	+	+	∞	∞	+	+	+	+
1/64	+	+	+	+	∞	∞	+	+	+	+
1/128	+	+	+	+	∞	∞	+	+	+	+
1/256	0	0	0	0	∞	∞	+	+	+	+

Experiment 7 is graphically represented in Text-fig. 4. An undoubted communal activity is apparent which bears a distinct relationship to the amount of the medium. It is seen that 400,000 bacteria placed in 5 cc. of medium multiplied and reached ∞ in 24 hours; the same number placed in 10 cc. of the medium had diminished to 240,000 at the end of 24 hours. Eventually conditions became uniform.



TEXT-FIG. 4.



TEXT-FIG. 5.

TEXT-FIG. 4, *a* and *b*. Graph of Experiment 7. (*a*) Series of tubes containing 5 cc. of broth each; (*b*) series containing 10 cc. each.

TEXT-FIG. 5, *a* and *b*. Graph of Experiment 8. (*a*) Series of tubes containing 3 cc. of broth each; (*b*) series containing 12 cc. each.

Experiment 8. Relation of Dose and Volume of Medium to Growth.—Strain 205, 24 hour culture. Centrifuged and resuspended in salt solution. Decreasing amounts inoculated into sets of four tubes of meat extract broth, two of 3 cc. and two of 12 cc.

Amount of culture.	Control plating (No. of colonies).	24 hr. readings.				Plating after 24 hrs.		48 hr. readings.			
		Tube No.				Tube No.		Tube No.			
		3 cc.		12 cc.							
		1	2	3	4	1	4	1	2	3	4
cc.											
1/2	∞	+	+	+	+	∞	∞	+	+	+	+
1/4	∞	+	+	+	+	∞	∞	+	+	+	+
1/8	∞	+	+	0	0	∞	∞	+	+	+	+
1/16	∞	0	0	0	0	∞	150,000	+	+	0	0
1/32	∞	0	0	0	0	650,000	15,000	0	0	0	0
1/64	∞	0	0	0	0	30,000	2,500	0	0	0	0
1/128	∞	0	0	0	0	10,000	2,000	0	0	0	0
1/256	2,000	0	0	0	0	400	360	0	0	0	0
1/500	3,000	0	0	0	0			0	0	0	0
1/1,000	1,000	0	0	0	0			0	0	0	0
1/2,000	60	0	0	0	0			0	0	0	0

This experiment is charted in Text-fig. 5. The result corresponds in a general way with that of Experiment 7 and shows that the growth phenomena are not in direct proportion to the amount of the medium.

The next experiments deal with the growth resulting from addition of several successive inocula to the same tube.

Experiment 9.—Strain 205, 24 hour culture, washed and diluted in salt solution and added in decreasing amounts to sets of two tubes of meat extract broth, 5 cc. per tube. To a third set of tubes 0.5 cc. of salt solution was added to make up to the volume of inoculated Sets 1 and 2, and all three sets were incubated together. A control plating was made of Set 1. After 24 hours samples (0.25 cc.) of Set 2 were plated, and then a second inoculation of a 24 hour culture of No. 205 similar to the first was made into Sets 2 and 3 while Set 1 received 0.5 cc. of salt solution. Set 1, therefore, was a control of the first inoculation, Set 3 was a control of the second inoculation, and Set 2 received two inoculations at intervals of 24 hours. At the end of 48 hours the first five tubes of all three sets were plated and counted. The actual numbers of viable bacteria per tube are set down.

First inoculation—start of experiment.			Counts at end of 24 hrs.			Remarks.
Tube No.			Tube No.			
1	2	3	1	2	3	
400,000	400,000	0	4,000,000*	4,000,000*	0	At this point the second inoculation of Tubes 2 and 3 was done.
200,000	200,000	0	120,000	120,000	0	
96,000	96,000	0	12,000	12,000	0	
48,000	48,000	0	5,000	5,000	0	
24,000	24,000	0	40	40	0	
12,000	12,000	0	40	40	0	
6,000	6,000	0	0	0	0	
550	550	0	0	0	0	
750	750	0	0	0	0	
400	400	0	0	0	0	
50	50	0	0	0	0	
10	10	0	0	0	0	
0	0	0	0	0	0	
9	9	0	0	0	0	
0	0	0	0	0	0	
0	0	0	0	0	0	

* Macroscopic growth.

Counts at beginning of second 24 hr. period.			Counts at end of second 24 hr. period.		
Tube No.			Tube No.		
1	2 (content = No. 1 + No. 3).	3	1	2	3
4,000,000*	4,640,000*	640,000	400,000*	∞*	∞*
120,000	440,000	320,000	200	∞*	100,000
12,000	172,000	160,000	10,000	360,000	27,000
5,000	85,000	80,000	220	50,000	1,500
40	40,040	40,000	3,000	1,600	500
40	20,040	20,000			
0	2,400	2,400			
0	800	800			
0	25	25			
0	5	5			
0	60	60			
0	30	30			
0	15	15			
0	1	1			
0	2	2			
0	5	5			

A study of this protocol clearly indicates synergistic activity. It is seen that doses of bacteria which in separate tubes were decreasing in number increased when brought together.

The last three experiments show in brief that, *ceteris paribus*, the minimal infecting dose of bacteria as defined in Experiments 4 to 6 varies not only with the total volume of the medium but depends also on whether the entire dose is received at once or in successive fractions. As indicated in Experiment 9, the matter is extremely complex and is evidently bound up with the question of variations in growth activity and latent period, but a test-tube analogy is furnished for the hypothesis (Dudley) that in human disease the infecting dose may be acquired fractionally and must reach a certain total concentration before growth occurs. This fact may have a bearing on the matter of adequate and potential contact which has been discussed above. It has been demonstrated experimentally that the greater part of a dose of bacteria placed in the upper air passages of human beings is rapidly eliminated within a few hours (12). Close association, with the acquisition not only of larger doses but of doses in more rapid succession, would accentuate the effect of summation and tend to emphasize the critical limit between adequate and potential contact.

Latent Period.

By lag, or the latent period in the growth of bacteria, "is meant the interval which elapses between the time of seeding and the time at which maximum rate of growth begins" (Chesney (13)). This biological phenomenon has been extensively studied and reference is made to the paper of Chesney (13) for the recent literature. In brief it may be said that when bacteria are transferred to a new medium the maximum rate of growth is not immediately attained but a preliminary period elapses during which there may be "slow growth, no growth, or an actual diminution of viable organisms." If, however, a culture is transferred to a suitable medium at the time of its most active growth there is no period of lag and multiplication immediately progresses at the most active rate. In the case of pneumococcus Chesney found the duration of the lag period to be about 4 to 6 hours if the transfer was made after the period of maximum growth had been passed.

As far as we know, the direct bearing on human infection of this phenomenon of lag has not been seriously considered. Yet it seems of the utmost importance as we have pointed out elsewhere (14). If one considers the course of an infection such as acute tonsillitis it is hardly possible to believe that the streptococci concerned maintain the same degree of vegetative activity throughout the disease. If a throat culture is made on the day before onset only a few streptococci may be recovered, but with the increase of the infection one immediately finds innumerable bacteria. Clearly there is extremely active growth at this time. After the subsidence of the acute disease fewer and fewer organisms are found and finally when a focal tonsillar carrier state is established few or no streptococci may be obtainable from the surface of the pharynx or tonsil and it may be necessary to aspirate the crypts to obtain the bacteria in question (15). It seems inconceivable that rapid growth is going on when the patient has reached the chronic carrier state. A sluggish multiplication probably continues in the depths of the tonsil and the organisms which are discharged to the outside world are certainly not in a highly vegetative state. Were they, there would be some evidence of surface multiplication on throat culture.

In the case of the respiratory tract there exists a highly effective mechanism for the elimination of bacteria which enter this region. This matter has been discussed in detail elsewhere. Suffice it to say that it has been shown that unless an active adaptation takes place within a few hours between the entering organisms and the mucous membrane of the host, the bacteria will be eliminated, passive persistance of germs in the upper air passages cannot occur for any length of time (16). It may be then that bacteria from a chronic carrier which enter the air passages of another person in a state of lag may be eliminated before active vegetative growth can be resumed. Such considerations might explain the evident difference in the number of infections which usually follows contact with active respiratory disease on the one hand and carriers on the other as illustrated for example by the epidemics of streptococcus respiratory disease which swept through the army camps.

The next experiments were designed to test the power of comparable numbers of bacteria, actively growing on the one hand, and in lag on the other, to produce test-tube infection. This was arbitrarily defined as visible growth in a standard tube of broth in 24 hours.

Experiment 10.—48 hour blood agar culture of Streptococcus 205 transferred to meat extract broth pH 7.4. 2 hours later, at a time when culture presumably was in lag, decreasing amounts were inoculated into sets of three tubes of meat extract broth. 2½ hours later a second set of inoculations was made.

Amount of 2 hr. culture.	Control plating (No. of colonies).	24 hr. readings. Tube No.			Amount of 4½ hr. culture.	Control plating (No. of colonies).	24 hr. readings. Tube No.		
		1	2	3			1	2	3
cc.					*cc.*				
1/4	1,088,000	+	+	+	1/4	1,433,600	+	+	+
1/8	544,000	+	+	+	1/8	716,800	+	+	+
1/16	272,000	+	+	+	1/16	358,400	+	+	+
1/32	136,000	+	+	+	1/32	179,200	+	+	+
1/64	68,000	+	+	+	1/64	89,600	+	+	+
1/128	34,000	0	0	0	1/128	44,800	+	+	+
1/256	17,000	0	0	0	1/256	22,400	+	+	+
1/500	8,500	0	0	0	1/500	11,200	+	+	+
1/1,000	4,250	0	0	0	1/1,000	5,600	+	+	+
1/2,000	2,050	0	0	0	1/2,000	2,800	+	+	+
1/4,000	1,500	0	0	0	1/4,000	1,600	+	+	+
1/8,000	800	0	0	0	1/8,000	1,200	0	0	0
1/16,000	600	0	0	0	1/16,000	550	0	0	0
1/32,000	400	0	0	0	1/32,000	200	0	0	0

It is seen that 68,000 bacteria from the lag culture was the minimum infecting dose whereas from the same culture 2½ hours later 1,600 bacteria produced visible growth in 24 hours.

Experiment 11.—Parallel experiments using 74 hour (lag) and 6 hour (active) culture of Streptococcus 205, in each case centrifuged and resuspended in salt solution.

6 hr. culture. No. of bacteria per tube.	24 hr. reading.	72 hr. culture. No. of bacteria per tube.	Readings.			
			24 hrs.	48 hrs.	72 hrs.	5 days.
∞	+	∞	+	+	+	+
∞	+	∞	+	+	+	+
∞	+	∞	+	+	+	+
∞	+	∞	+	+	+	+
∞	+	∞	+	+	+	+
∞	+	∞	0	+	+	+
∞	+	∞	0	+	+	+
∞	+	28,000	0	+	+	+
∞	+	7,000	0	+	+	+
40,000	+	5,000	0	0	+	+
28,000	+	4,800	0	0	+	+
7,000	+	2,500	0	0	+	+
3,500	+	1,100	0	0	+	+
1,800	+	600	0	0	+	+
1,500	+	500	0	0	0	+
700	+	400	0	0	0	+
650	+	300	0	0	0	+
500	+	250	0	0	0	+
500	+	210	0	0	0	+
400	+	175	0	0	0	+

In this experiment as few as 400 organisms from an active culture produced infection in 24 hours whereas innumerable viable bacteria (as determined by preliminary plating) from a culture in lag failed to infect. At the end of 5 days, however, growth had occurred in all the lag tubes.

In both Experiments 10 and 11 a relatively unfavorable medium was employed. With meat infusion broth which is highly favorable for this organism no striking difference in growth could be demonstrated between 4 hour, 48 hour, and 4 day cultures; all of them produced infection in minimal amounts (Experiments 1 to 3).

At this point reference must again be made to Experiment 4 (Text-fig. 1). It is seen that an initial inoculation of 80,000 bacteria led to

no growth and the plating yielded no colonies at 24 hours. With an initial inoculation of 160,000 the count was 8,000 at 24 hours and 5 at 48 hours. During the next 24 hour period, however, the surviving 5 bacteria multiplied with great speed and macroscopic growth with innumerable colonies was attained at 72 hours. There was clearly some marked difference in the state of growth activity of the 80,000 bacteria, which all died in 24 hours, while 5 survivers in the same medium were eventually able to grow.

Similar phenomena referable to variations in growth activity are evident throughout the entire series of experiments and probably play an important part in Experiment 9.

If a state of affairs analogous to that brought out in the preceding experiments holds in human infection certain problems are perhaps made clear. In Experiment 11 there were required to produce infection thousands of times more bacteria in lag than from an actively growing culture. If the organisms harbored by chronic carriers are in lag it is apparent why, under average conditions of contact, infection does not spread, whereas an epidemic of acute cases with actively vegetative organisms once started leads to progressive spread even with the same conditions of exposure of susceptibles. On this basis one may readily explain the start of an epidemic without assuming any preliminary alteration in virulence of the bacteria, a consideration which has always been a puzzle in epidemiology.

One may compare an outbreak of tonsillitis with an epidemic of milk-borne septic sore throat. In the latter case the incidence of infection among those drinking the infected milk is high; at the same time the organisms can in no sense be regarded as specially adapted to a human environment. They are derived from the infected udder of the cow whence they are discharged into the milk. However, when the human being drinks such infected milk he receives not only a huge dose of streptococci but also streptococci which are in a favorable medium and in a state of very active growth. On the other hand, in the case of tonsillitis, a disease caused by apparently identical organisms, despite the fact that infection is spread directly from person to person the cases remain sporadic in type even when the community is replete with carriers. But again epidemics of streptococcus respiratory disease may occasionally occur though only under conditions of extreme crowding or when susceptibility is generally lowered by a previous infection such as influenza or measles.

It seemed of interest to produce in the test-tube an "epidemic" starting from a "carrier" without needing to assume alterations in "virulence" of the bacteria. The following experiment was planned. To represent the chronic carrier from which the epidemic was started an old culture of Streptococcus 205 was used. Tubes of meat extract and meat infusion broth represented relatively susceptible and insusceptible people. A 6 hour culture in meat infusion broth represented an organism actively growing as in a case of an acute disease.

Experiment 12.—(*a*) A carrier is in a community of relatively susceptible (A) and insusceptible (B) people. Under average conditions of contact Dose *X* is the greatest amount received by any one person per unit of time. Infection must take place within 24 hours or it is assumed that the bacteria are eliminated.

Dose (No. of colonies) (carrier culture).	Tubes (B) (24 hr. readings).									
	1	2	3	4	5	6	7	8	9	10
400 = *X*	0	0	0	0	0	0	0	0	0	0
300	0	0	0	0	0	0	0	0	0	0
250	0	0	0	0	0	0	0	0	0	0

Result, no infection; corresponds to usual natural conditions.

(*b*) Effect of crowding (increase in dose to *Y* times *X*).

Dose (carrier culture).	Tubes (B) (24 hr. readings).									
	1	2	3	4	5	6	7	8	9	10
500,000 (*Y X*)	+	+	+	+	+	+	+	+	+	+
250,000	0	0	0	0	0	0	0	0	0	0
100,000	0	0	0	0	0	0	0	0	0	0

Result, certain individuals receive a dose so great that even with an inactive strain infection is produced.

(*c*) Recently infected individuals now distribute active strain. Even if crowding is abolished and the greatest amount received per unit of time falls again to *X* this dose may infect.

Dose (active culture).	Tubes (B) (24 hr. readings).									
	1	2	3	4	5	6	7	8	9	10
400	+	+	+	+	+	+	+	+	+	+
300	+	+	+	+	+	+	+	+	+	+
250	+	+	+	+	+	+	+	+	+	+

Result, epidemic.

(*d*) The same effect as crowding (*b*) may be brought about by introducing an unusually susceptible individual into the community who can be infected by Dose *X* as follows:

Dose (carrier culture).	Tubes (B).										Tubes (A).	
	1	2	3	4	5	6	7	8	9	10	1	2
400 = *X*	0	0	0	0	0	0	0	0	0	0	+	+
300	0	0	0	0	0	0	0	0	0	0	+	+
250	0	0	0	0	0	0	0	0	0	0	+	+

SUMMARY.

In the preceding experiments an attempt has been made to work out on a mathematical basis certain laws of "test-tube infection" with special reference to the relation of dosage and activity of culture to resulting growth in favorable and unfavorable media. It has been shown that neither lag nor dosage is of great importance when dealing with a highly favorable medium, but that with an unfavorable medium both determine very directly the outcome of the inoculation.

Certain analogies have been drawn between these test-tube results and the observed phenomena of human and animal infection. If conditions are actually similar in the latter there is a simple explanation of several of the puzzles of epidemiology. It becomes intelligible why a carrier of pathogenic bacteria may reside in a community without producing infection of the susceptibles about him in spite of the fact that they actually acquire the organisms, why crowding results in the spread of infection, and why the introduction of unusually susceptible people into a community where carriers are present leads not only to infection of these susceptibles but to infection of the general population.

The experiments further raise the query whether alterations in natural vegetative activity of bacteria as well as specific alterations in virulence are of importance in explaining the spread of infection.

Finally, it should be said that the present experiments are not regarded as evidence for any hypothesis of human infection. They merely furnish interesting analogies. Further work with animals along the same lines is in progress.

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OBSERVATIONS ON BLOOD REACTION IN EXPERIMENTAL PNEUMONIA.*

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It has been previously reported (1) that we succeeded in obtaining experimental pneumonia in 46 of a series of 60 dogs inoculated by tracheal insufflation with various organisms. Of these 46, eight died within 18 hours of inoculation, and two were inadequately observed, so that satisfactory data were obtained from 36. Detailed results on six of these, inoculated with *Bacillus mucosus capsulatus*, were presented (1), in which erythrocyte counts, blood volume, hemoglobin, and hematocrit determinations revealed an actual loss of circulating erythrocytes, without compensatory regeneration, during the course of the pneumonic process.

It is the purpose of the present paper to report certain data obtained from the remaining 30 of the successfully observed animals. This number includes eight inoculated with *Bacillus bronchisepticus*, one with *Streptococcus hæmolyticus*, one with *Streptococcus viridans*, one with *Bacillus influenzæ*, and nineteen with *Bacillus mucosus capsulatus*. In these animals, lung lesions were followed by fluoroscopic and x-ray examinations; lung ventilation was recorded, when the dogs were not too large, by means of a body plethysmograph, and 20 cc. of blood, on the average, were drawn for the various blood determinations. Data were secured daily in these ways until death or recovery of the animals took place.

The blood was drawn from the femoral artery into an oxalated syringe through the intact skin, with suitable aseptic precautions and measures to prevent hemorrhage. Determinations of the alkaline

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reserve and of the total acetone bodies were made on most of the dogs by essentially the methods introduced by Van Slyke, Cullen, and Fitz (2–4). In four we followed the oxygen content of arterial and venous blood, using Van Slyke's technique (5), and in five animals we studied the carbon dioxide content of arterial blood by the method of Van Slyke and Stadie (6). In these five dogs, we also made electrometric measurements of the pH of the arterial blood. In five other dogs, quantitative estimations were made of the urea and ammonia content of the urine and qualitative tests were made for the presence in the urine of albumin, sugar, acetone, and diacetic acid. Whenever blood was used for gasometric determinations, it was drawn out of contact with air, and transferred immediately under oil to the instruments in which the measurements were made. Except for the acetone bodies, determinations were made in duplicate.

RESULTS.

In Table I data are submitted showing the effects of the pneumonia produced by the various organisms on the lung ventilation, alkaline reserve, acetone bodies, and erythrocyte and leucocyte counts, in typical animals selected from the series. In Table II results may be found for two dogs inoculated with *Bacillus bronchisepticus*, and two with *Bacillus mucosus capsulatus*, with respect to lung ventilation, alkaline reserve, acetone bodies, and oxygen content of arterial and venous blood. Table III presents the significant figures on lung ventilation, acetone bodies, carbon dioxide content of arterial blood, and the hydrion concentration of arterial blood in five dogs inoculated with *Bacillus mucosus capsulatus*. Finally, the urine findings, together with data on the alkaline reserve, in three typical animals inoculated with *Bacillus mucosus capsulatus*, may be seen in Table IV. Detailed results are presented, therefore, on eighteen of the thirty animals. The findings on the twelve animals omitted from the tables closely parallel the data shown, according as to whether death or recovery took place, but the estimations on the various functions were not as complete as in the case of those included in the tables.

The results varied with the virulence of the infection, and with the extent and duration of the pulmonary involvement. As was noted previously (1), the most constant finding was a reduction in the

TABLE I.

Data upon Lung Ventilation, Alkaline Reserve, Acetone Bodies, Erythrocytes, and Leucocytes, in Dogs with Experimental Pneumonia.

Dog No.	Date.	Lung ventilation per min.	Alkaline reserve.	Acetone bodies per liter.	Erythrocytes per c.mm.	Leucocytes per c.mm.	Organism; x-ray findings; remarks.
	1920	*cc.*	*vol. per cent*	*mg.*	*million*	*thousands*	
4	Oct. 6	3,818	40.1	36.2	6.4	8.2	*B. bronchisepticus.* Pulse 156.
	" 8	4,967	41.4	25.8	6.2	37.5	Right middle lobe diffuse. Pulse 190.
	" 11	7,918	41.2	90.6	5.4	38.8	Entire right lung dense. Pulse 150.
	" 12	2,513	37.6	178.1	5.4	40.2	Right lung dense. Pulse 156.
	" 14	2,378	39.8	160.2	5.1	21.8	Right lung diffuse. Pulse 150.
Recovery.	" 18	3,689	40.2	76.3	5.6	18.1	Right middle lobe diffuse. Pulse 130.
6	" 18	6,361	46.4	53.1	6.6	9.3	*B. bronchisepticus.*
	" 20	4,783	39.2	88.3	5.2	19.6	Right lung diffuse.
	" 22	5,135	33.8	75.8	4.4	32.2	" " dense; lower left diffuse.
	" 25	4,520	47.4	66.9	3.6	51.0	Both lungs dense.
Death.	" 27		31.2	188.4		78.4	Marked lipemia.
12	Nov. 16	2,861	42.1	21.7	6.4	16.2	*B. bronchisepticus.*
	" 17	2,720	53.2	48.6	6.2	33.4	Right middle dense.
	" 19	6,050	46.9	35.2	5.6	30.4	Left upper diffuse.
	" 21	2,639	46.1	24.3	5.8	27.2	Right middle "
Recovery.	" 23	3,028	46.3	37.0	5.1	26.0	Both lungs clear.
16	Dec. 21	2,025	45.4	33.5	5.8	14.3	*Streptococcus hæmolyticus.*
Recovery.	" 23	2,816	43.0	53.2	5.2	31.0	Right middle diffuse.
	1921						
20	Jan. 10	6,038	46.8	15.3	8.7	10.1	*Streptococcus viridans.*
	" 12	5,107	43.5	44.6	5.6	29.8	Both upper lobes dense.
Recovery.	" 14	5,430	51.4	65.0	6.8	17.2	Right middle diffuse.
22	" 18	2,680	55.0	23.0	6.4	18.2	*B. influenzæ.*
	" 20	4,702	42.3	67.1	4.9	36.1	Right lower diffuse.
	" 22	1,124	42.8	88.9	4.8	42.4	Entire right " left upper dense.
Recovery.	" 25	3,122	50.1	43.0	6.0	46.5	Right lung clear; left upper diffuse.

number of circulating erythrocytes, which occurred in all animals, but most noticeably in those in which the pneumonia was severe and of long duration. In most cases there was an increase in lung ventilation during the first few days of the disease, which was followed

TABLE II.

Data upon Lung, Ventilation, Alkaline Reserve, Acetone Bodies, and Oxygen Content of Arterial and Venous Blood in Experimental Pneumonia.

Dog No.	Date.	Lung ventilation per min.	Alkaline reserve.	Acetone bodies per liter.	Arterial oxygen.	Venous oxygen.	Organism; x-ray findings; remarks.
	1920	*cc.*	*vol. per cent*	*mg.*	*vol. per cent*	*vol. per cent*	
8	Nov. 3	5,249	43.0	19.2	24.2	18.4	*B. bronchisepticus.*
	" 6	4,920	39.0	68.5	16.8	12.3	Right lung dense.
Death.	" 7	2,909		121.7			" " very dense; left upper dense.
9	" 6	3,866	43.6	29.7	22.3	17.5	*B. bronchisepticus.*
	" 8	5,975	50.9	32.3	18.4	12.0	Left lower diffuse; right upper diffuse.
	" 9	3,138	44.8				Entire left dense.
	" 10	2,614	33.7	167.0	15.3	9.9	Left dense; right diffuse.
	" 12	4,017	46.5	28.1	16.5	9.4	" lung diffuse.
Recovery.	" 15	4,112	47.2	11.5	16.4	12.2	Lungs clear.
		Respirations per min.					
15	Dec. 8	20	41.8	32.6	19.6	14.8	*B. mucosus capsulatus.*
	" 10	40	32.4	103.1	17.5	12.3	Right lung dense.
Recovery.	" 13	60	42.3	60.4	17.4	10.3	Lungs clear.
	1921						
29	Feb. 11	20	50.8	46.3	20.7	15.6	*B. mucosus capsulatus.*
	" 12	32	49.0	44.8	19.7	12.8	Left lung diffuse.
Death.	" 13	16	39.5	133.6	15.2	9.0	" dense; right upper diffuse.

by a marked decrease in many instances in which the lung lesions were severe and widespread. If recovery took place, the lung ventilation returned to normal or went above normal, as the lungs cleared. In the acute and fatal cases, the respiratory activity was usually markedly diminished.

The alkaline reserve showed significant loss only in the most severe or fatal cases. There often appeared to be a mobilization of alkali in the blood during the first few days following inoculation, which in one instance (No. 12) was marked and continued during the course of the infection. A lowering of the alkaline reserve due to increased

TABLE III.

Data upon Lung Ventilation, CO_2 Content and pH of Arterial Blood, Acetone Bodies, and Erythrocytes in Dogs Inoculated with B. mucosus capsulatus.

Dog No.	Date.	Lung ventilation per min.	Arterial CO_2.	pH	Acetone bodies per liter.	Erythrocytes per c.mm.	Organism; x-ray findings; remarks.
	1921	*cc.*	*vol. per cent*		*mg.*	*million*	
31	Feb. 15	4,300	51.3	7.41	68.0	7.2	
Death.	" 17	3,220	48.7	7.33	93.6	6.0	Entire left dense.
32	" 18	3,100	43.5	7.46	37.2	6.8	
Death.	" 20	2,850	38.7	7.32	89.3	5.6	Entire left and right middle dense.
35	" 23	3,320	57.5	7.52	18.2	7.8	
	" 25	4,610	42.4	7.42	40.1	6.9	Entire left and right upper diffuse.
Recovery.	" 26	3,180	43.2	7.42	42.6	6.8	Left lower diffuse.
37	Mar. 1	2,530	44.2	7.43	38.6	7.8	
	" 3	2,940	38.8	7.31	61.7	6.2	Left upper dense.
Death.	" 4		36.4	7.25	102.4	6.0	
38	" 2	2,280	40.4	7.47	26.5	7.2	
	" 4	3,070	38.2	7.39	53.8	6.6	Left lower dense; right middle diffuse.
Death.	" 5	1,720	24.1	7.22	122.3	6.0	Entire left dense; entire right diffuse.

pulmonary activity, according to the acapnial process as outlined by Henderson and Haggard (7), was noted in four animals (Nos. 16, 22, 35, and 38). The significantly low levels of alkaline reserve, however, usually coincided with high levels of acetone bodies. The acetone bodies generally increased when lung ventilation was reduced,

and when the pulmonary involvement was most severe. In light cases, as in No. 12, the changes in the acetone bodies were relatively insignificant.

Arterial and venous oxygen diminished as the infection followed inoculation, and if recovery took place, the values tended to return gradually to the normal. The diminution was in relatively the same proportions for arterial and venous blood, within the limits of error, so that it is doubtful if any marked change took place in the rate of oxygen consumption. The tendency of the figures indicates, however, a slight increase. We made no determinations of oxygen capacity,

TABLE IV.

Data upon Alkaline Reserve, Quantitative Urea and Ammonia, and Qualitative Albumin, Sugar, Acetone, and Diacetic Acid, in Experimental Pneumonia.

Dog No.	Date.	Alkaline reserve.	Urea per 24 hrs.	Ammonia per 24 hrs.	Albumin.	Sugar.	Acetone.	Diacetic acid.
	1921	*vol. per cent*	*gm.*	*gm.*				
41	May 20	42.3	0.252	0.102	−	−	−	−
	" 22	38.6	0.421	0.232	+	−	−	−
Death.	" 24	33.2	0.529	0.308	+	−	+	−
43	July 12	46.1	0.305	0.127	−	−	−	−
Death.	" 13	34.0	1.340	0.572	+	−	?	?
44	" 14	47.3	0.210	0.085	−	−	−	−
Death.	" 15	38.8	1.440	0.408	+	−	?	−

so that no conclusions can be drawn from our figures regarding the oxygen unsaturation of arterial or venous blood.

The hydrion concentration of arterial blood, as indicated by pH, rose after the inoculations, the most marked increases being noted in the fatal and prolonged cases.

The daily urea and ammonia output showed a considerable rise after inoculation. Albumin was noted in the urine in all cases examined, and it appeared continuously during the course of the infection. Sugar was not found at any time. Acetone was definitely noted in the urine in only one instance, while diacetic acid was absent except in one doubtful case.

DISCUSSION.

An explanation of these changes in the blood and urine in experimental pneumonia may be offered on the basis of reduced oxidation. The anoxemia in pneumonia is of both the anoxic type, as defined by Barcroft (8), due to the pulmonary congestion in the lungs, and of the anemic type, as evidenced by the reduction in erythrocytes and hemoglobin (1). By interfering with normal oxidative processes in the body, the pneumonic anoxemia may lead to an increased pulmonary ventilation, with blowing off of carbon dioxide, and a consequent disturbance in the acid-base relationship, or if the respiratory mechanism cannot meet the emergency, to an accumulation of fixed acids in the blood with a resulting acidosis.

These experimental observations confirm many clinical reports.

Peabody (9) noted a decrease in the carbon dioxide of blood in lobar pneumonia, and Palmer and Henderson (10) found that during the fastigium of pneumonia larger amounts of sodium bicarbonate by mouth were necessary to lower urine acidity than was the case in normal conditions. Lewis (11) reported that the blood of pneumonic patients had a diminished affinity for oxygen, which is characteristic of acidosis. Palmer (12) found that a large amount of organic acid, free at a pH of 5.0, was excreted in the urine of cases of acute lobar pneumonia, but that the acidosis, as indicated by the combined carbon dioxide in the plasma, was seldom severe. Barach, Means, and Woodwell (13) observed that the available blood alkali in pneumonia, as shown by the level of the carbon dioxide dissociation curve, tended to be lower than the normal. It was reported by Killian (14) that about the time of the crisis in pneumonia of various types, there was an increase in the non-protein nitrogen, uric acid, urea, and creatinine in the blood, with a decrease in the chlorides and carbon dioxide-combining power.

Our experimental findings also lend support to the rationale of the oxygen and alkali therapy in pneumonia as developed by Barach and Woodwell (15) and by Means and Barach (16), and of the oxygen chamber treatment of pneumonia as introduced by Stadie (17). It was suggested by Barach, Means, and Woodwell (13) that when an acidosis was present in pneumonia patients, either in the sense of a low level of available blood alkali or a diminished blood pH or a combination of the two, the administration of sodium bicarbonate might help to reduce the work of the respiratory system. It was pointed out that the use of the alkali should be carefully controlled in order

to avoid an alkalosis, and that when anoxemia is present, oxygen therapy should also be used. It would seem from our data that a possible acidosis in pneumonia would be dependent upon a developing anoxemia, and that the alleviation of this should be the first consideration.

It has been stated by Barach (18) that: "The disease in which acute anoxemia occurs with the greatest frequency and with the greatest severity is pneumonia." Our study has indicated the rapidity and severity with which this anoxemia may be produced under experimental conditions. It is possible roughly to correlate our indications of developing acidosis with the severity of the pulmonary development as revealed by x-ray examinations, but with the exception of the rapidly fatal cases, almost the same correlation may be made with the severity of the anemia. It is more likely, however, that the acidosis of pneumonia is due chiefly to the anoxic anoxemia, and that the anemic anoxemia secondarily contributes to its development. Light might be shed upon this question by determining the oxygen capacity of arterial blood during the course of experimental pneumonia. Unfortunately, we made no such determinations, but the evidence from clinical studies does not indicate that the anemic anoxemia is of very great importance in the development of the acidosis of pneumonia.

There is no evidence from our figures that the anoxemia encountered in pneumonia may be of the stagnant type. This agrees with the report made by Stadie (19) on the patients studied by him, in which the oxygen consumption remained within normal limits, indicating that the cardiac output had not been diminished. In these clinical cases, Stadie (19) found no unusually low oxygen capacities for the blood, but a tendency for an increased arterial and venous oxygen unsaturation. An arterial unsaturation of more than 20 per cent was usually associated with a fatal outcome. These findings indicate that the anoxic anoxemia in pneumonia is relatively far more marked than the anemic anoxemia.

SUMMARY.

The correlation of the lung lesions and anemia in dogs suffering from experimental pneumonia induced by various organisms, with

changes in the respiratory activity and in the acid-base relationship in blood, indicates that the developing acidosis, most marked in the severe cases, is due chiefly to the effects of an anoxic anoxemia.

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OBSERVATIONS ON SOME CAUSES OF GALL STONE FORMATION.

III. The Relation of the Reaction of the Bile to Experimental Cholelithiasis.

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In previous papers it has been shown that there exists a well defined tendency for calculi to form out of the sterile liver bile of dogs, irrespective of whether or not these animals are losing the secretion;[1] and that after hepatic injury of various sorts potential centers for lithiasis are frequently abundant therein.[2] Why, then, are gall stones almost never encountered in autopsies on dogs dying from ordinary causes? We have suggested an answer to this question in so far as it concerns the ducts. There are several evident safeguards against stone formation within them,—their motility, the cleansing and possibly antagonistic action of the secretion they themselves elaborate, and the flushing out effected by an intermittently quickened bile stream. But how is one to explain the absence of calculi from the gall bladder, a viscus which, according to the latest evidence, empties itself but poorly, and in which the bile often undergoes great concentration[3] and must frequently be held for many hours together. Nearly half of the score or more of normal canine gall bladders that we have examined contained an abundant débris consisting of shreddy mucous particles and flakes of long desquamated epithelium not infrequently stained black as result of secondary changes in pigment during the course of their sojourn in the organ. Once actual quartz sand was present,—small granules resisting aqua regia, like those not infrequently recovered

[1] Rous, P., McMaster, P. D., and Drury, D. R., *J. Exp. Med.*, 1924, xxxix, 77.
[2] Rous, P., Drury, D. R., and McMaster, P. D., *J. Exp. Med.*, 1924, xxxix, 97.
[3] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 47.

from the biliary system of other domestic animals,[4] into which they were presumably forced from the intestine by the muscular contractions of the gut. Yet never have gall stones been present. In view of the marked tendency for calcium carbonate to come down out of liver bile on standing, and for it to deposit within old organic débris,[1] it seems certain that the normal gall bladder must either effect some change in the secretion in the direction of safety, or else add some preventative against deposition. Both possibilities are within reason, since the *vesica fellea* is known to alter markedly the fluid coming to it.

Influence of the Gall Bladder on the Bile Reaction.

The solubility of calcium carbonate, the substance most abundant in the gall stones that we have induced experimentally in dogs, is greatly affected by the reaction of the fluid in which it is contained. Lichtwitz[5] has touched upon the possible influence of the bile reaction on gall stone formation in his discussion of the puzzling absence of calcium bilirubinate from normal human bile. Naunyn[6] before him had tentatively advanced the view that a special reaction may be necessary for the formation of the substance. Following the line of thought suggested by our own work, and by such speculative reasonings, we have tested the reaction of bladder bile of the dog and have compared it with that of the secretion as derived from the hepatic tissue. To our surprise bladder bile proved frankly acid to litmus in a large proportion of instances, whereas liver bile was nearly always somewhat alkaline, and usually markedly so. The literature showed that the difference had already been noticed by Okada[7] for the dog, and by Neilson and Meyer[8] for the rabbit. Curiously enough, it has failed to attract attention in its possible bearing on gall stone formation.

Okada used the capillary electrometer. He ascertained that the pH of the gall bladder bile of dogs ranged from 5.43 to 6.97. His animals almost without exception had been fasting; but the data he obtained in other species supported

[4] Kitt, T., *Lehrbuch path. Anat. Haustiere*, Stuttgart, 3rd edition, 1906, ii.

[5] Lichtwitz, L., *Ergebn. inn. Med.*, 1914, xiii, 1.

[6] Naunyn, B., cited by Lichtwitz.[5] See also Naunyn, B., *Mitt. Grenzgeb. Med. u. Chir.*, 1921, xxxiii, 1.

[7] Okada, S., *J. Physiol.*, 1915–16, l, 114.

[8] Neilson, N. M., and Meyer, K. F., *J. Infect. Dis.*, 1921, xxviii, 511.

the view that bladder specimens may be alkaline, acid, or neutral, with a tendency for them to be relatively acid after fasting. Liver bile, procured through a fistula of Schwann, was, by contrast, always alkaline, with a range in pH of 7.54 to 8.15. Fasting was without an evident influence upon such specimens. Those exposed to air rapidly became more alkaline.

Neilson and Meyer made use of a colorimetric method and studied rabbit bile especially, but some other biles also. They corroborated Okada's findings and traced the change in pH on exposure to air to a loss of CO_2. They showed further that base-forming diets notably increase the average titrable alkalinity of rabbit bile, whence they inferred that this alkalinity is due to the presence of carbonates.

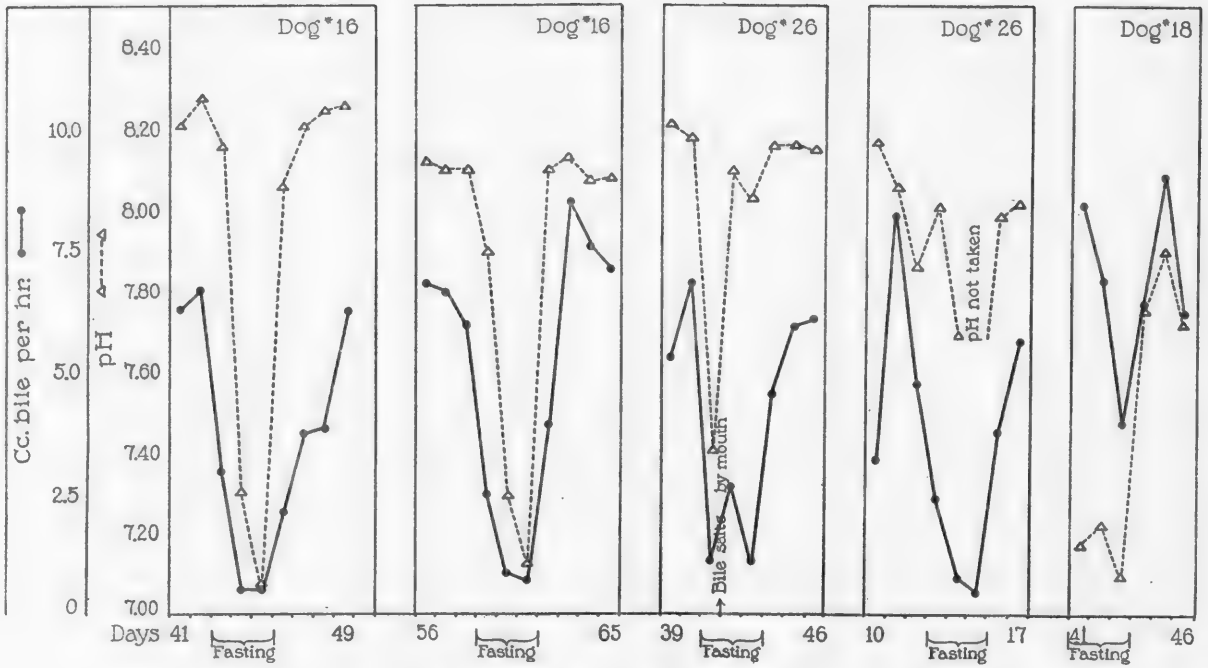
We have employed the potentiometer. The readings were made in duplicate at 37°C. Exposure of the specimens to air was avoided by withdrawing them from the collecting balloons, or from the gall bladder, directly into Luer syringes cleansed of alkali by long previous use and boiled in water for present purposes. The determinations were made as soon as possible after the bile had been procured, though tests showed that even several hours in the syringe did not notably alter the pH.

Day-to-day pH determinations were made on the liver bile of twelve dogs studied with special reference to the bile sediment. The observations in some instances extended over many weeks. In addition, the total calcium content of the bile was determined as routine by ashing, precipitation with sodium oxalate, and titration with potassium permanganate. The results of the analyses will form the topic of a separate communication. They will be considered here only in so far as they bear directly on the problem under discussion.

The liver bile regularly proved alkaline, often more strongly so than Okada found. The pH ranged from 7.07 to 8.55 and tended to approximate 8.20 ordinarily. But Okada's specimens, unlike ours, had been subjected to gall bladder influence, owing to the circumstance that the organ formed a link in the fistula system. On the other hand some carbonate precipitation had occurred in our specimens during their period of sojourn in the collecting balloons. This should have rendered the bile less alkaline, if anything, owing to the fact that calcium carbonate represents the union of a strong base with a weak acid. The precipitate was present in proportionate amount in the samples tested.

Fasting is attended by a great diminution in the amount of bile secreted, and in a marked and in some instances closely reciprocal increase in the concentration of certain of its constituents.[9] According to Okada, the reaction undergoes no change. But we find to

[9] McMaster, P. D., Broun, G. O., and Rous, P., *J. Exp. Med.*, 1923, xxxvii, 395.



All biles sterile except that of Dog 18.

TEXT-FIG. 1. Influence of fasting on the amount and pH of liver bile.

the contrary that the bile regularly becomes less alkaline during a fast, and, as this progresses, may become approximately neutral to litmus, though the pH has never in our experience quite reached 7.00 (Text-fig. 1).

The gall bladder bile of a number of normal dogs was procured by operation under ether. As in the case of liver biles, the individual differences in reaction were greater than those noted by Okada,—which would naturally follow from the larger and more various material at our disposal. The gall bladder specimens obtained a few hours after a meal were either neutral to litmus or alkaline—not infrequently as alkaline as some liver biles,—whereas those procured after 48 hours of fasting, were as a rule markedly acid and reddened litmus promptly. Specimens of this latter sort were heavily pigmented and syrupy, appearing greatly concentrated. It has long been supposed on good evidence [10,11] that during intervals between meals bile accumulates in the gall baldder and is concentrated and stored there, to be more or less gradually voided and replaced with new when the digestion of a meal takes place. Recent observations which indicate that the viscus empties itself imperfectly do not invalidate this view. Fresh support for it is to be found in our observations. In normal dogs recently fed, the reaction of the gall bladder contents approximated that of liver bile, whereas after fasting it was found acid, and concentrated and thickened, all to a degree never encountered in liver bile, as procured from fistulas. That the prevailing alkalinity of liver bile is not due to the loss of secretion entailed by the fistula may be safely inferred from the alkaline character of the gall bladder contents of full fed normal animals (Table I).

The alteration that takes place in the reaction of bile within the gall bladder may conceivably be the result of chemical changes occurring in a labile secretion as result of its prolonged retention at body heat, or on the other hand, of some influence exerted by the organ of retention. That the latter is the true explanation may be inferred from the fact that liver bile collected into a balloon and kept warm remains highly alkaline, as further from the demonstrated activity of the gall bladder

[10] Naunyn, B., *Arch. Anat., Physiol., u. wissensch. Med.*, 1869, 579.

[11] Babkin, B. P., Die äussere Sekretion der Verdauungsdrüsen, Berlin, 1914, 344.

to influence the bile in other ways, and from Neilson and Meyer's[8] observation that in gall bladders so injured by infection as to lose the function just referred to, the pH of the bile remains that of the secretion as derived from the liver.

Influence of the Bile Reaction on Carbonate Precipitates.

Granting, then, that the gall bladder acts to render the bile acid, it has remained to demonstrate an influence of changes in the reaction of dog bile upon carbonate precipitation. This has been sufficiently accomplished by a series of simple experiments with bile that contained such a precipitate. They need be but briefly described.

TABLE I.

The pH of Gall Bladder Bile and Its Relation to Fasting.

Dog No.	pH	Remarks.
1	5.18	No food for 24 hrs. and but little at last feeding.
2	5.67	
3	5.41	
4	6.20	No food for nearly 24 hrs. and long ether anesthesia.
5	5.31	
6	5.57	
7	7.05	No food for nearly 24 hrs.
8	6.82	
9	6.27	
10	7.06	Food within a few hrs.
11	7.43	
12	7.33	
13	7.61	
14	7.03	
15	7.00	
16	7.55	

Several like portions of a single specimen of dog bile, each with its share of crystalline carbonate, as obtained from the collecting balloon, were distributed in 50 cc. Pyrex centrifuge tubes previously rinsed with distilled water. Some were rendered neutral to litmus, and others made faintly acid thereto, while still others were kept as controls. The acids employed for the purpose were N/10 hydrochloric acid, concentrated lactic acid, and 3 per cent acetic acid. The

specimens treated with hydrochloric acid became opalescent owing to the development of a finely divided mucous precipitate, but the others appeared unchanged. All were now brought to the same bulk by the addition of distilled water, and either centrifuged at once or allowed to stand overnight at room temperature before so doing. The experiment was several times repeated. The controls regularly yielded the sediment of carbonate originally present, whereas in the portions made neutral or acid it was completely or partially dissolved. Some of the original specimens contained pigmented nuclei, potential centers for lithiasis, of the sort heretofore described,[2] as well as carbonate crystals. These nuclei dissolved, but somewhat less rapidly than the associated carbonate crystals.

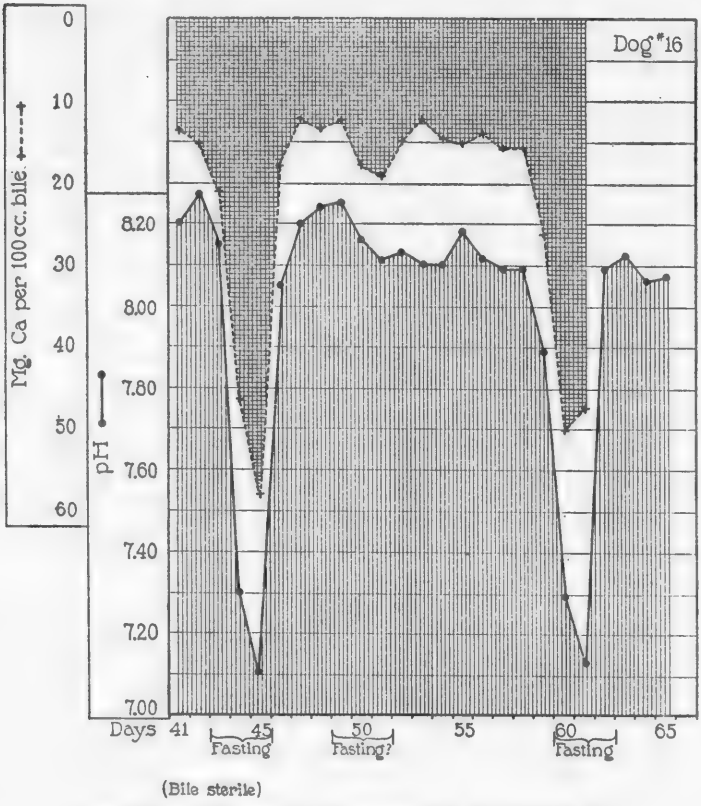
Several unsuccessful attempts were made to determine a precipitation of calcium carbonate out of liver bile by exhausting it of free carbonic acid. For the purpose, the secretion was taken from the collecting balloon into large centrifuge tubes filled with CO_2 gas, deprived by centrifugation of the sediment previously formed, separated into portions under CO_2, and some of these portions placed *in vacuo* for several hours, with the container tilted so that a large surface of fluid was exposed. No carbonate came out, nor did it in the controls. Evidently the precipitation of the salt occurring in the balloon had reduced the bile content of it far below the saturation point.

Efforts to dissolve a carbonate sediment by bubbling CO_2 through the bile were likewise unsuccessful, probably because the period of a few hours allotted to the tests was too short. The greatest change accomplished was a dubious rounding of the crystal edges. The results have an interest in connection with the not infrequent presence of carbonate calculi within glass bulb connections interpolated in the course of the common duct of animals otherwise normal, which connections must have been flushed at intervals with more or less acid bile from the gall bladder. The persistence of the calculi may be laid to the transitory nature of this flushing. The bulbs when removed from the animals always contained alkaline bile.

It would seem from these experiments, crude though they are, that no other explanation is needed for the lack of carbonate deposition in the normal gall bladder than the changes in reaction of the bile effected by this viscus. Further observations upon the changes occurring in the liver bile during a fast, and on those in the bile that assembles and distends the gall bladder after obstruction of the common duct clearly indicate that the reaction of the secretion undergoes an alteration in the direction of safety when there might otherwise be danger of carbonate precipitation.

The Calcium Concentration and Reaction of the Bile as Affected by Fasting.

On the withdrawal of food from the intubated dog for 2 or 3 days, the bile output usually lessens greatly, and may diminish to less than one-tenth of the ordinary amount.[9] Such alterations are accompanied



TEXT-FIG. 2. Calcium concentration and pH of liver bile.

by a reciprocal increase in the concentration of bilirubin, and the total output from day to day remains the same. There is a marked but less considerable increase in the calcium per cc. (Text-fig. 2). And *pari passu* with this increase and the pronounced slowing of the bile stream,

changes both that, other things being equal, would favor precipitation within the duct system, there occurs a change in the reaction of the bile such as would tend to rule out this possibility. The pH falls from an average of approximately 8.20 to close to 7.00, that is to say to approximately the neutral point for litmus (Text-figs. 1 and 2). The precipitate found in balloon specimens collected under these circumstances is often far less than the ordinary, and never greater despite the high concentration of the bile.

The Calcium Concentration and Reaction of Stasis Bile.

When the common duct of a previously normal animal is obstructed a very great concentration of the bile pent in the bladder may come about through the action of this viscus.[12] Yet, as is well known, stones fail to form out of the dark, thick fluid.

We have, in seven instances of experimentally induced stasis, ascertained the reaction of the bladder bile and determined its bilirubin and calcium content.

Obstruction was produced by tying and cutting the common duct in every instance save one in which it followed the clotting of blood within a glass tube which had been interpolated in the duct. Asepsis was maintained during the operations and the eventual stasis fluid regularly proved sterile on culture. It was taken into syringes under the precautions already described. For comparison with it the gall bladder content was removed at the initial laparotomy through a rubber catheter run up into the viscus by way of a slit made in the common duct below the point at which this was to be ligated. The dogs had purposely been fed full a few hours before operation. Thereafter they as a rule ate fitfully, if sometimes well.

From the table (Table II) it will be seen that the period of obstruction ranged from 2 to 6 days. The eventual bladder contents showed, in comparison with that originally procured, a pigment concentration which was sometimes nothing less than startling. In the case of Dog 1, there was 18 times as much pigment per cc. in the stasis specimen as in the normal sample originally had, and in Dog 6, 53 times as much. Part of this increase is attributable to the influence of operation on the character of the bile[9] but most of it is undoubtedly due to the con-

[12] Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1921, xxxiv, 75.

centrating activity of the *vesica fellea*.[3] The change in calcium concentration was, relatively speaking, negligible, though in four out of five instances there was a well defined increase, once to double the initial amount. No calcium precipitate was ever found. The pH of the initial specimens has been either alkaline or neutral, whereas that of the stasis bile was acid, sometimes notably so.

TABLE II.

The Reaction and Calcium Content of Stasis Bile.

Dog No.	Stasis.	Bile character.	Bilirubin per cc.	pH	Total calcium per 100 cc.	Remarks.
	days					
1	0	Watery, clear, yellow-brown.	0.47	7.61	22.8	Fed on day of operation.
	3	Greenish black.	8.42	6.79	59.0	
2	0	Pale amber, clear.	0.56	7.55		Fed on day of operation.
	5	Brownish black.	4.66	5.82	53.3	
3	0	Amber, clear.	0.46	7.0	38.0	Fed on day of operation.
	6	Greenish black.	2.2	6.1	44.6	
4	0	Dark brown, flaky.	1.03	7.03	28.5	Fed but ate little on day of operation.
	4	Dark brown.	1.78	6.85	21.8	
5	0	Pale yellow.	0.3	7.43	30.7	Fed on day of operation.
	2	Greenish black.	4.26	6.15	47.4	
6	0	Pale yellow.	0.18	7.06	30.7	Fed on day of operation.
	2	Greenish black.	9.61	6.14	53.0	
7	?	Greenish black.	0.78	5.18	39.7	Tissue icterus, so obstruction had probably lasted at least 1 week.

All the bile specimens proved sterile.

The changes in reaction which occur in normal bladder bile, in the liver bile of fasting animals, and in stasis bile take place at times when the calcium content of the bile is increased. That they are not an indirect result of such increases is sufficiently shown by the lack of correspondence between the two under conditions of stasis (Table II).

Gall Bladder Shortcomings and Stone Formation.

If changes in bile reaction effected by the gall bladder act as the normal preventative of carbonate lithiasis, when these changes are suppressed stones should form. We have been spared the labor of experiments on the point through the existence in the literature of observations which suffice to illustrate it and which are the more telling because recorded in another connection.

Meyer, Neilson, and Feusier[13] have noted that in rabbits with a typhoid infection of the gall bladder, stones consisting of calcium carbonate regularly develop when the animal survives for a considerable period (100 days). They attribute the calculi, as have others in the past, to the inflammation set up by the infection, and they believe that all carbonate stones are traceable to infection, a view rendered untenable by our work.[1] In a separate paper, Neilson and Meyer have dealt exhaustively with the reaction of normal and pathological bile but in no reference to stone formation. They state that the liver bile of the rabbit is regularly alkaline, having a pH of 7.6 to 7.9, whereas that from normal gall bladders may be acid, neutral, or alkaline, the pH ranging between 5.7 and 7.7, much as in the dog. The bladder biles of typhoid carrier rabbits were, by contrast, alkaline with the pH never less than 7.00 and on the average approximating that of normal liver bile. They conclude that "the gall bladder of a 'carrier' animal has apparently lost its concentrating function and therefore fails to change the reaction of its content." They mention that some of the carrier biles they tested contained carbonate sediment.

In brief, these observations clearly show that gall bladders which have been damaged by the typhoid bacillus fail to alter the character, and in special the pH, of the bile coming to them from the liver. When this abnormal state of affairs endures for some time a carbonate lithiasis inevitably develops. That there is a relationship of cause and effect between the bladder shortcomings and the development of stones cannot be definitely affirmed. Yet the findings bring support by the method of difference to the conception,—based initially on evidence through the method of agreement,—that the formation of carbonate stones in the gall bladder of the dog and rabbit is prevented under normal conditions by changes in the reaction of the bile that the organ effects.

[13] Meyer, K. F., Neilson, N. M., and Feusier, M. L., *J. Infect. Dis.*, 1921, xxviii, 456.

The Reaction of the Bile in Its Relation to Human Cholelithiasis.

Stones formed predominantly of calcium carbonate are rare in man. Human calculi are of such varied character that a single factor can scarcely be the prime agent in the genesis of all. Yet the question is worth asking whether the reaction of the bile, or to speak more concretely, the failure of a pathologically damaged gall bladder to alter the reaction of the bile, may not have a share in determining human lithiasis.

In a preceding paper[2] we have described spheroliths of calcium carbonate occurring in the sterile bile from human gall bladders containing calculi, and serving as centers for a formation of secondary stones. It has been interesting to compare the reaction of biles containing such spheroliths with that of specimens in which they were not present. Nine samples of bile in all have been tested, which were removed from the gall bladder at operation, and also subsequent fistula specimens from four of the cases. The derivation and general characters of the material have been described in the paper just referred to. We are indebted for the specimens to Doctor Allen Whipple of the College of Physicians and Surgeons of Columbia University.

The bladder biles were aspirated direct from the organ into Luer syringes previously submitted to boiling water; and the drainage specimens were collected, under oil occasionally, in Pyrex centrifuge tubes. The patients had received no food on the day of operation. While care was exercised to prevent exposure of the bladder contents to air, in most instances this was not wholly prevented. Dog or rabbit bile becomes more alkaline on contact with air, and some alteration in the same direction may be expected in the human instance.

When the amount of bile was sufficient its reaction was determined in duplicate with the potentiometer; but frequently reliance had to be placed on litmus.

In two out of four instances of cholelithiasis with mild chronic cholecystitis the bladder bile had a pH of 7.00 and of 6.98, respectively; and in one of the others,—the sole one of them with an infected bile,—it was neutral to litmus, and in the fourth alkaline. The bile from a peculiarly shaped but otherwise normal gall bladder had a pH of 6.72; that from a case with dubious cholecystitis in the absence of infection a pH of 7.28; while in an instance of mild chronic inflammation with infection, the pH was 7.06. The sterile white bile associated with a carcinoma of the head of the pancreas that had metastasized to the

gall bladder wall was markedly acid, having a pH of 6.29; whereas in another instance of a growth localized as yet to the pancreas but producing intermittent biliary obstruction the tarry specimen proved alkaline to litmus, possibly as result of the presence of blood intermixed with it when procured. There must have been little, if any, impairment of the concentrating activity of the bladder in this last case. All of the fistula specimens were highly alkaline, their pH averaging 8.00. But they were collected during the adjustment period after obstruction, while the patient was still jaundiced, and hence must be regarded as abnormal.[9] Also there had been a relatively great exposure of them to air.

The reaction of human bile has received much notice in the past, though usually of a perfunctory character. The secretion is stated to be either alkaline or neutral to litmus. Little distinction has been made between gall bladder and duct specimens, and some authors do not mention the indicator that they employed. Neilson and Meyer have cited the literature. They alone made pH determinations, on fistula specimens from a single patient. They found that the pH from day to day approximated 8.00. They do not mention precautions to prevent exposure of the material to air.

An immediate conclusion which follows upon the inspection of our scanty data together with the prior ones of Neilson and Meyer is that many more cases must be studied, and under conditions better controlled, before certainty will be reached as concerns the effect of the gall bladder upon the reaction of the bile. But the available data show at least that the gall bladder bile of man is in some instances neutral or slightly acid to litmus, whereas liver bile tends to be alkaline. And the findings as a whole support the supposition that the secretion is alkaline as it comes from the liver, but that it undergoes an alteration toward the acid side during its sojourn in the *vesica fellea*, just as in the rabbit and the dog.

Changes in Reaction Determining Precipitation from Human Bile.

Is the solubility of the substances out of which human stones form affected by slight changes in the bile reaction? We have made some simple experiments on the point.

Experiment 1.—Into each of five small test-tubes, there was put 1 cc. of a medium brown bile with pH of 7.00 from a gall bladder containing "old" cholesterol stones. The fluid was sterile but slightly cloudy, because of the distribution through it of fine, myelin-like bodies, as the microscope showed. No sediment came down from it on centrifugation. To four of the tubes were added respectively 1, 2, 3, and 4 drops of N/10 potassium hydrate in water. All were now allowed to stand overnight at room temperature and again centrifuged. From the tube to which nothing had been added and from that containing 1 drop of potassium hydrate there was recovered a copious, light brown sediment of myelin-like material which dissolved almost completely in acetone and proved on test to be mainly cholesterol. The supernatant bile was alkaline to litmus in both instances. Acidification of it with HCl failed to cause the sediment to go into solution again. In the tubes containing 2 to 4 drops of hydroxide, no precipitation had occurred.

Experiment 2.—A bile, dark brown, thin, and faintly cloudy, from a case of chronic cholecystitis, was rid of a slight mucous sediment by centrifugation and distributed in ¾ cc. portions in each of four small test-tubes. The fluid had been sterile, with a pH of 7.06. To one portion a drop of 1 per cent lactic acid was added, to others, 1 and 2 drops of potassium hydrate, respectively, while a fourth was kept as such. All were allowed to stand at room temperature for 3½ hours and then centrifuged. The acid specimen yielded no sediment, whereas that kept as such gave a slight one of mucus and myelin bodies (cholesterol), while those containing potassium hydrate yielded a well marked one of the same materials.

Experiment 3.—A sterile, medium brown, clear, and slightly syrupy bile from a case of dubious cholecystitis was centrifuged to rid it of a few cells which it contained; and 1 cc. specimens were placed in each of three test-tubes. The initial pH of the fluid was 7.28. To one tube a drop of 1 per cent lactic acid was added, to another a drop of N/10 potassium hydrate, while the third was left as such. After 1½ hours at room temperature all were centrifuged, sedimentation noted, and they were let stand overnight and centrifuged again. No further sediment had come down during the interval. In the tube receiving alkali there had been present, after the initial hour and a half, a considerable deposit of cholesterol in myelin form, and in the tube let be as such, a slight sediment of the same material, whereas in that containing acid no deposition whatever occurred.

Another experiment similar to that just described, carried out with a ropy, black bile, failed of result in the way of sediment, as did one with a thin, lightly pigmented, and presumably highly dilute, fistula specimen secreted during the readjustment period after relief from obstruction.

Our finding, that small quantities of base or acid will determine or prevent a precipitation of cholesterol from human bile, might perhaps have been predicted from the known complexity of the secretion and,

in special, from that of the relationships whereby cholesterol is maintained in its distribution through the fluid. Yet the results do not lose in signficance for this reason.

Human bile is a well buffered fluid; and the quantity of hydrate or acid added in our tests cannot have greatly altered the reaction. Neilson and Meyer neutralized some rabbit biles with concentrated lactic acid or N/10 HCl and found that there occurred only a temporary change in the hydrogen ion concentration which in some instances returned to the initial figure within 24 hours. Others before us have noted that cholesterol frequently falls out of human specimens that have stood for a few hours *in vitro*. An explanation for the phenomenon may be found in alkali derived from the glassware; and another in a presumptive loss of carbon dioxide on exposure of the bile to air. Whatever the cause for the deposition, it has, in our experience, proved preventable through the addition to the bile of a trace of acid. On the other hand, bile rendered acid to litmus may have no effect to dissolve a cholesterol deposit that has once formed. Certainly it had none in the single test we made.

In three of our instances of cholelithiasis there was present in the gall bladder a quantity of bile sand in addition to large stones. Fragments of the older stones together with cholesterol plates made up the bulk of this sand in two of the cases; but there were present as well, in all, minute concretions, spheroliths which consisted of calcium carbonate in impure form. These have been described in detail in a previous communication.[2] They were beautifully concentric in structure, and on the surface of some of them a deposition of cholesterol, or of pigmented amorphous material, or of both had taken place, while still others were situated deep within larger stones. Their interest in the present connection lies in their association with a heavily pigmented, thick bile, one that is to say which had undergone alterations within the gall bladder. For it may be recalled that in the case of the dog and rabbit carbonate precipitation tends to take place from the unchanged liver bile on standing; and we have referred the absence of precipitation within the gall bladder to the activities of this viscus to prevent it by altering the bile. There can be no doubt that in the human instances now under discussion one of these activities, namely that through which concentration of the bile is effected, had still

been retained in considerable part by the pathologically altered bladder; while that the ability to alter the reaction of the secretion had not been wholly lost seems probable from the fact that one specimen containing spheroliths was neutral to litmus and another had a pH of 6.98. The third bile was faintly alkaline to litmus.

The circumstances under which the spheroliths originally formed are of course unknown. We have already drawn attention to the fact that carbonate concretions may not readily redissolve in bile,—this while discussing their presence upon the walls of glass tubes interpolated in the common duct of dogs and intermittently flushed with normal bladder bile. It is possible as accounting both for their origin and survival in our human cases that there was a pathological increase in the bile content of calcium; or that the concentration of the secretion effected by the gall bladder was of an abnormal sort, preventing solution of the spheroliths despite favorable changes in the bile reaction, or that these changes themselves were never sufficiently great to accomplish this. Speculation upon the point is idle. But the findings themselves serve to forecast a difficulty which may be expected in attempts to correlate the reaction of human bile with the presence of stones.

DISCUSSION.

The problem of cholelithiasis presents two major facets to the pathologist, why stones begin, and why they grow. In a previous paper we have considered some aspects of that first mentioned and have shown that in the dog, at least, potential opportunities for stones to begin are offered in many sorts of liver disturbance. Out of the bile elaborated under such pathological conditions there come down "showers" of little concretions, nuclei which can serve as centers of stone formation, and which frequently did so under the abnormal circumstances of our experiments. Potential nuclei of other sorts, organic débris in special, must pass along the biliary channels at some time or other in the life of many individuals.

In the present communication we have been concerned with why stones grow, or, to put the problem in a form more accessible to attack, why, given the recurring opportunities just mentioned for stones to arise they remain rare, relatively speaking. The question is an old one,

if stressed anew by our observations. For the statement has often been made, with a query appended, that gall stones seldom form on the sediment so frequently contained in the gall bladder; and the fact has repeatedly been noticed that although foreign bodies frequently serve as the nuclei of gall stones, their presence in the biliary tract is not of itself sufficient to cause lithiasis.[14]

The experiments and observations here recounted afford an explanation for the occurrence of calcium carbonate stones in laboratory animals which is more than merely rational being probably the real one. A study along similar lines of the reaction of the bile in its possible relation to the development of calculi consisting of calcium bilirubinate would appear to be highly desirable, because of the important rôle conceded to the substance in human stones. But the indications are that this may prove difficult. For the physical chemistry of bilirubinate precipitation is a complicated matter; while furthermore the methods at present available do not always enable one to identify the substance with certainty in the test-tube. It is deposited from the bile of the dog only on special occasions and then in small amount.[1] The existing literature on stones of the guinea pig and rabbit suggests that in these species as well it is seldom formed in quantity.

According to the thesis supported by our work, carbonate stones develop in intubated dogs and in typhoid carrier rabbits as the result of a tendency for the salt to be deposited out of the normal bile of both species. In healthy animals of the sorts mentioned this tendency is counteracted in the gall bladder by a change that the organ effects in the reaction of the bile, as result of which carbonate remains in solution despite the great diminution its fluid vehicle undergoes. When the gall bladder function is so impaired that it fails to effect this change and the reaction of the bile remains what it was when first derived from the hepatic tissue, carbonate lithiasis may automatically ensue.

Does this explanation of the occurrence of a certain type of calculi under controlled conditions in some laboratory animals bring with it any implications as regards the causes for human cholelithiasis? Needless to say, a single factor can scarcely be expected to account for

[14] Mignot, R., *Arch. gén. méd.*, 1898, x, series 8, 129.

all the diverse categories of human stones. Yet the fact is worth recalling that calcium carbonate is no uncommon constituent of these stones, if seldom a principal one. As already mentioned, we have recovered calcium spheroliths from human gall bladders in which they were serving as centers for a secondary formation of gall stones. The suggestion that calcium bilirubinate may be precipitated from human bile at a certain reaction only was made years ago.[5] And the test-tube findings of the present work sufficiently prove that a precipitation of cholesterol out of the bladder bile of man can be determined or prevented *in vitro* by slight additions of base or acid which would tend to effect alterations in the bile reaction such as there is reason to suppose may be decisive within the organism. These are suggestive facts. Our purpose is not to emphasize them but to outline, irrespective of them, an attitude toward the problem of human cholelithiasis which may perhaps have advantages besides novelty. It has become old habit to ask, why do stones form? But might not the question better be, why are they ever absent? May not a gall bladder or duct functionally impaired through infection or by other means (recurring stasis?) become *particeps criminis* to stone formation as result of its sins of omission? Faulty evacuation of the bile channels has long been deemed a contributary element in lithiasis. But may not a failure on the part of the gall bladder to modify the bile committed to it be sometimes the decisive factor?

To test the hypothesis in its bearing on the human case will require a large material. Quite possibly the normal gall bladder effects other changes in the bile besides those of reaction, that are inimical to stone formation, serving in this way to offset the favoring influences of concentration and retention. But much remains to be learnt in relation to the influences of the bile reaction. It is needful to ascertain first of all whether, as our findings seem to indicate, the normal gall bladder of man effects changes in the reaction of its content comparable with the changes occurring in the dog and rabbit. In these days of "prophylactic" ablation, data on the point will not be difficult to procure. But to determine whether stones form when the gall bladder fails to alter the reaction of the bile may prove an arduous task. A few of the difficulties have already been touched upon in connection with the figures on the pH of human biles containing carbonate spero-

liths. The problem was simpler in the case of typhoid carrier rabbits, because of the limiting conditions and the type of calculus encountered. But in patients coming to operation, the manifestations of physiological principles are only too often overlaid by phenomena irrelevant to them, for example those of infection. That the human gall bladder, though containing stones, may retain part at least of its ability to concentrate the bile is certain. Our findings suggest that the ability to alter the bile reaction may likewise not be totally lost under these circumstances. Fitz[15] has concluded as the result of a series of observations upon operative material, that bladder bile from gall stone patients tends to be less pigmented, of lower specific gravity, and less rich in nitrogen than that from cases of cholecystitis only. The finding might perhaps be taken to suggest, in line with our hypothesis, that a failure of the bile reservoir to modify the secretion coming to it from the liver is concerned in the development of stones. But the need of the moment is for data.

Conjectures only can be made as to the means whereby alterations in the reaction of the bile come about in the *vesica fellea* of the dog and rabbit. It is reasonable to suppose that the same processes of osmosis and diffusion may be responsible which bring about a reduction in the bulk of the secretion. Were this the case, one would expect to find an impairment of the concentrating ability constantly associated with a greater or less failure to alter the bile reaction,—unless indeed the permeability of the bladder wall for some substances may be altered without affecting that for others.

SUMMARY.

As previous papers from our laboratory have shown, there exists a well defined tendency for calcium carbonate to come out of solution in the normal liver bile of the dog, and for it to be deposited on certain nuclei not infrequent in the secretion under pathological circumstances. Gall stones that had arisen in this fashion were a frequent occurrence in the intubated animals we studied. The present paper is concerned with the reasons for the absence of such stones from dogs with an intact biliary tract.

[15] Fitz, R., and Aldrich, M., *J. Am. Med. Assn.*, 1922, lxxix, 2129.

The solubility of calcium carbonate is known to be markedly affected by the reaction of the fluid in which it is contained. The normal liver bile, out of which it tends to precipitate, is alkaline, with an average pH of 8.20 but in the gall bladder where conditions might otherwise seem especially favorable to precipitation, the secretion undergoes a change toward the acid side, becoming on long sojourn there, strongly acid to litmus (pH 5.18 to 6.00). From bile as thus altered, no carbonate precipitation takes place, even when it becomes greatly concentrated as in fasting animals or after obstruction of the common duct. Furthermore, carbonate which has precipitated out of liver bile on standing dissolves again in it when the fluid is rendered slightly acid *in vitro*, or, in some cases merely neutral to litmus.

There are several obvious reasons for the absence of carbonate stones from the normal ducts under ordinary conditions,—notably the motility of these latter, the flushing that they undergo from an intermittently quickened bile stream, and the cleansing and possibly antagonistic action of the secretion elaborated by the duct mucosa. In the fasting animal, one at least of these influences is almost done away with, the rate of bile flow is so greatly cut down; while furthermore the calcium concentration of the secretion undergoes a considerable increase. But *pari passu* with these changes there occurs one in the bile reaction, a diminution in alkalinity so great that the pH often approximates that of the neutral point for litmus. That this change is not a direct consequence of the increase in calcium, may be inferred from the findings with stasis bile, the calcium content and reaction of which were observed to vary independently, if in general in the same direction.

These adjustments within the organism, some of which may be thought to exhibit an element of the purposeful, when considered with the test-tube experiments, strongly suggest that the reaction of the bile plays a critical part in determining the occurrence of carbonate stones, as furthermore that their absence from the normal gall bladder is a consequence of the changes in the bile reaction there occurring. The changes come about through a functional activity of the bladder. This being the case, one might suppose that the failure to act would be followed by a formation of carbonate stones. There is sufficient evidence available in the literature to indicate that this happens, in rabbits at least.

It is important to know whether changes in the bile reaction play any part in determining the cholelithiasis of man. To determine the matter will require a large material. But this much we have shown, that carbonate spheroliths not infrequently serve in human beings as centers in a formation of secondary stones of carbonate and cholesterol, as further that cholesterol precipitation out of human bladder bile can be induced or prevented by slightly altering the reaction of the fluid toward the alkaline and acid sides, respectively.

The possibility that cholelithiasis may be a consequence of sins of omission on the part of the biliary channels and reservoir deserves to be considered.

THE LIVER REQUIREMENT OF THE FASTING ORGANISM.

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PLATE 21.

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It is a truism among physiologists that the parenchymatous organs contain far more functional tissue than is required for immediate body needs. In the case of the liver, as much as three-fourths of the total parenchyma can be removed without eliciting other derangements than those occasionally ensuing through interference with the passage of the portal blood.[1] There always follows, though, a rapid regeneration from the fragment left and soon the organ has reached its old proportions. The tendency to the maintenance of these proportions is demonstrable by other means. If no ablation is done but merely a local diversion of the portal flow so that a part of the liver receives all of the blood from this source and the remainder none, the favored tissue rapidly increases in amount by cell proliferation while that deprived atrophies and disappears to a corresponding extent. The shifting of tissue thus entailed, which eventually becomes complete, is so ordered throughout that the total liver bulk at any one time approximates that of the entire normal organ. More striking still, the atrophy fails to occur, at least in any considerable degree, when a compensating hypertrophy is prevented by experimental methods.[2] The organism would seem to require just so much liver, no less and no more, and this much it preserves despite the profound circulatory derangement.

Ponfick believed that the regeneration which takes place after the removal of hepatic tissue is consequent on a functional demand or stimulus; and there is every reason to suppose that this is the true cause and the cause as well for the balanced shifting of tissue just

[1] Ponfick, E., *Virchows Arch. path. Anat.*, 1889, cxviii, 209; 1890, cxix, 193.

[2] Rous, P., and Larimore, L. D., *J. Exp. Med.*, 1920, xxxi, 609.

described. One need only recall the functional hypertrophy of other organs, as *e.g.* of a kidney after removal of its fellow. But granting that the stimulus is functional, that recurring body needs force the liver, so to speak, to be big, what is the nature of these needs? Can a single one of the tasks accredited to the organ be responsible in the matter of liver size, or does this result from a summation of demands? As the first step in an attempt to obtain a better understanding of the problem thus defined, we have made observations on the liver requirement during prolonged fasts,—under conditions, that is to say, which rule out all functional stimuli connected with the assimilation of food by way of the digestive tract.

Choice of Animals.

The size of the normal liver is notoriously subject to individual variation and to variation as result of the physiological incidents of daily life. The data from many instances are required for a clear demonstration on any factors affecting it. One must perforce use small animals. The herbivora are obviously unsuitable, if experiments on the effect of special diets are to be carried out. By process of exclusion the rat and mouse are come to as the species of choice; and of these the rat is obviously preferable, by reason of size and hardiness, and because of the numerous statistical data on record concerning it.

Changes in the liver of the rat take place with much greater rapidity than in large animals, as might perhaps be expected from its relatively brief life cycle and the generally swift pace of events within its body. For example, the complete shift of hepatic tissue occurring after local portal diversion as above described, which in the dog requires 6 months or more for completion, and in the rabbit, 2 months, takes in the rat only about 2 weeks, as we have recently found. Needless to say a great advantage of time is gained by the utilization of animals in which adjustments occur so quickly.

Method.

Since this paper is introductory to further work by the same method, we shall deal at some length with operative procedure, factors of error, and so forth.

Adult male white rats from the Institute breeding room have been employed almost exclusively. The weights as a rule ranged between 150 and 200 gm. For most of our purposes, they have been kept on wood shavings in individual jars, and fed the ration employed with success in breeding, namely an abundance of bread and milk each morning and of grain each afternoon with a small piece of dog biscuit every few days.

For the experiments on inanition here to be reported, glass jars equipped with a water bottle and having at the bottom a raised wire dais,—merely the cover of another, smaller jar,—were employed. The dais had a sufficiently coarse mesh for the feces to fall through. It and the jar were washed daily. When placed in metal metabolism cages, fasting rats waste quickly, presumably from loss of heat, and are liable to die out of hand, but in jars of glass and in a room kept at about 80°F. as ours was, they lose weight gradually and remain vigorous for from 7 to 12 days, depending on age and individual state. A close oversight was kept upon them all. The routine was to chloroform them while in good condition. Throughout the fasting period they drank copiously, so the factor of inspissation can scarcely have entered into the results.

Complicating Diseases.

Few intercurrent disorders occur to complicate observations upon the rat liver. The most obvious is the encysting of *Tænia crassicollis*, but this is rare in animals kept caged from birth as most of ours were. A more serious difficulty is presented by the liver enlargement associated with "giant spleen." Statistical observers have noted that rats with abnormally large spleens are prone to have abnormally large livers as well;[3] and we can corroborate their finding. The nature of the hepatolienal complex seems not to have been studied and it is highly obscure. The hepatozoon of Miller has been absent in our cases; the liver appeared normal histologically; and there were no striking microscopic changes in the spleen. Even in the fasting animal the liver remains very large (*vide* that of Rat 8, Table I). Fortunately, the disease, if such it be, was seldom encountered in our stock. All rats in which it was recognizable at autopsy were ruled from account in the experiments; yet there can be no doubt that mild forms of the disorder would have escaped notice.

Many white rats, no matter how carefully fostered, sooner or later acquire "lung disease," a chronic, spreading consolidation; and this constitutes a principal obstacle to their use, as previous workers have found. Animals in which the disease has a foothold can often be recognized for rejection by the presence of a sanguineous discharge about the nostrils, as well as by palpable râles. In the Institute stock it has been relatively infrequent and few of our experiments have been complicated by it. When just beginning, as an atelectatic consolidation of a lung lobe, usually the azygos, or as a few minute, scattered, fleshy grey nodules it does not notably affect the general condition, nor, in our experience, the liver size.

[3] Hatai, S., *Am. J. Anat.*, 1913–14, xv, 87.

TABLE I.

Changes in the Liver Weight of the Fasting Rats of Experiment 6.
(a) Changes in the Whole Liver of Laparotomized Controls.

Rat No.	Body weight.				Liver weight.					Body condition.	Liver condition.	Remarks.
	Initial.	At operation.	Final.	Loss.	Initial (calculated).	Final.		Final total in per cent of.				
						Total.	Lobe mass.	First body weight.	Last body weight.			
	gm.	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*					
1	180	177	133½	25.8	7.39	4.43	1.33	2.46	3.32	Rather thin.	Slightly atrophic.	No real emaciation.
2	178½	171½	128½	28.0	7.32	3.96	Not taken.	2.22	3.08	Thin.	Markedly atrophic.	
3	166	164½	108½	34.6	6.81	2.4	0.78	1.45	2.21	Extreme emaciation.	Markedly atrophic.	
4	177	169½	113½	35.9	7.26	3.42	1.12	1.93	3.01	Marked emaciation.	Moderately atrophic.	Azygos lobe of right lung in atelectatic consolidation.
5	163½	157	103	37.0	6.71	3.18	1.03	1.95	3.09	Marked emaciation.	Markedly atrophic.	Considerable atelectatic consolidation in lungs.
6	180	169	111	38.3	7.39	2.59	Not taken.	1.44	2.33	Marked emaciation.	Markedly atrophic.	
7	186	175½	114½	38.4	7.63	2.25	0.66	1.21	1.96	Marked emaciation.	Markedly atrophic.	
Average.....				34.0				1.81	2.71			
8	151	139	91½	39.4	6.19	3.82	1.17	2.53	4.17(!)	Marked emaciation.	Markedly atrophic.	Ruled out because of "giant spleen."

TABLE I.

Changes in the Liver Weight of the Fasting Rats of Experiment 6.
(b) Changes in the Liver Remnant of the Ablation Animals

Rat No.	Body weight.				Liver removed.	Weight of lobe mass.					Body condition.	Liver condition.	Remarks.
	Initial.	At operation.	Final.	Loss.		Initial (calculated).	At operation (calculated).	Final.	Final weight in per cent of. First body weight.	Final weight in per cent of. Last body weight.			
	gm.	*gm.*	*gm.*	*per cent*	*gm.*	*gm.*	*gm.*	*gm.*					
9	163	160	115½	29.2	3.35	2.08	1.52	3.65	2.24	3.16	Marked emaciation.	Hypertrophic; slightly fatty.	
10	176	174½	123½	29.8	3.51	2.25	1.59	3.47	1.97	2.81	Marked emaciation.	Hypertrophic; slightly fatty.	
11	170	162	112½	33.8	3.4	2.18	1.54	3.31	1.95	2.94	Moderate emaciation.	Hypertrophic; not fatty.	Atelectatic consolidation of upper quarter of left lung.
12	150	143	94	37.4	3.02	1.92	1.37	2.57	1.71	2.73	Marked emaciation.	Hypertrophic; not fatty.	
13	180½	171	112	37.9	3.6	2.31	1.63	2.63	1.46	2.35	Very marked emaciation.	Slightly hypertrophic.	
14	166	158	96½	41.8	3.73	2.13	1.71	2.09	1.27	2.17	Extreme emaciation.	Normal.	Moribund when killed.
15	150	142	81½	45.7	3.83	1.92	1.75	1.72	1.15	2.11	Extreme emaciation.	Slightly atrophic.	Hematuria; died; autopsied prior to clotting of blood.
Average.....				36.5					1.68	2.61			

Explanation of Table.—The initial body weights of the table were obtained after a 6 hour fast. There was a further fast of some 18 hours prior to operation, involving a not inconsiderable further loss of weight.

The initial liver weights in the control series is calculated on the basis that 4.1 per cent of the body weight consisted of hepatic tissue; and the initial weight of the lobe mass on the further assumption that of the hepatic tissue 31.2 per cent lay in said mass.

Measurements.

The length of the rat from nose to anus has regularly been ascertained since it yields with the weight some index to the nutritive condition of the animal. Well nourished, but not fat, individuals were chosen for the work. Immediately after the final chloroforming the livers were taken out, and in consequence they were obtained in a relatively bloodless state. The point is an important one because when autopsy is delayed but a few minutes, much blood remains in the hepatic tissue after excision despite the circumstance that clotting has not yet occurred. In the lack of other, more satisfactory criteria, the gross weight of the liver was of necessity taken as the indicator of the true amount of the organ present in the body, meaning by true amount that of parenchyma. It is known that liver tissue may on occasion become transiently swollen with glycogen or fat, as further that its protein content may be altered by the feeding of protein.[4] During digestion the organ may be 12 per cent larger than between whiles.[5] In order to lessen the error from these sources in establishing a normal weight for the liver, the healthy rats to be examined were deprived of their usual ration on the day on which they were to be killed, and were sacrificed about 6 hours after this feeding would ordinarily have been given. The stomach was regularly found empty at this time and the intestine nearly so; while the liver was rather sharp edged and flabby with little if any fat visible histologically and almost no glycogen. The gross body weight now closely approached what Jackson[6] calls the net body weight (gross body weight less that of the intestinal contents). Little was to be gained by waiting longer, since the intestinal contents underwent only a negligible decrease in bulk during a further fast of 18 to 24 hours. Throughout inanition, as Jackson has found, the intestines retain material to the amount of some 2 to 3 per cent of the gross body weight. We have not attempted to determine the figure in each of our animals but have been content with the gross weight.

Effects of Inanition on the Liver.

In human beings and lesser animals receiving water but no food, the liver undergoes a remarkable diminution in size. Only the adipose tissue loses more in weight. The differing loss by various organs has given ground for the belief that those suffer least which are essential to the body economy, as, for instance the heart.[7] Voluntary muscles that are frequently used waste less than those unem-

[4] Tichmeneff, N., *Biochem. Z.*, 1914, lix, 326.

[5] Howell, W. H., Text-book of physiology, Philadelphia and London, 6th edition, 1917.

[6] Jackson, C. M., *Am. J. Anat.*, 1915, xviii, 75.

[7] Aschoff, L., *Path. Anat.*, 1911, i, 41.

ployed. Manasseïn[8] holds, in accordance with these facts, that the liver changes result from a lessening functional demand which is consequent on the fasting. Paschutin,[9] on the other hand, has propounded the view that the various tissues lose during inanition in proportion as they are themselves available as food. The difference in opinion brings up a crucial point as regards the relation of the liver to the body needs. Has this portal organ any great uses during inanition? Will not an inconsiderable fraction of it suffice under such circumstances? It has seemed to us possible to obtain an answer to these questions by a simple experiment. By operation one can summarily reduce the body content of hepatic tissue to less than the amount persisting in advanced inanition of the intact animal. When now food is withdrawn from rats treated in this way, what will happen? If the reduction in size of the liver that occurs ordinarily during inanition is the result of a utilization of the organ as food then one might expect a dwindling of the fragment left at operation; but if the liver size is determined by functional needs then perhaps the fragment will hypertrophy, despite the general food lack, to meet the necessities of the fasting body.

The liver of the rat, as of the cat, dog, and rabbit consists of two masses of unequal bulk each with its own vessels and ducts, connected by an inconsiderable isthmus of parenchyma. In the rat the larger mass, or "main liver," lying between stomach and diaphragm, contains, as we have found in twelve normal animals, an average of 68.8 per cent of the total hepatic tissue. The operative removal of this mass is well borne. The average for the smaller moiety, or "lobe mass," lying above the right kidney, is, as would follow for the figure given above, 31.2 per cent and the greatest variations noted were 25.7 and 34.7 per cent, respectively. The individual figures in most instances came remarkably close to the average. According to Jackson[6] rats in extreme inanition still have some 42 per cent of liver tissue left. There is reason to suppose the figure to be an unduly low one. For the normal weights on which Jackson's calculation of the amount of change was based were those of organs from animals well fed to the time of death, and having for that reason an hepatic parenchyma containing much extrinsic material. In such rats the livers constituted 4.3 to 4.5 per cent of the total body weight, whereas

[8] Manasseïn, W., Dissertation, St. Petersburg, 1869, abstracted by Mühlmann, M., *Centr. allg. Path. u. path. Anat.*, 1899, x, 160.

[9] Paschutin, W., *Vorlesungen über allg. Path.*, pt. 2, St. Petersburg, 1881, cited by Mühlmann, M., *Centr. allg. Path. u. path. Anat.*, 1899, x, 160.

in ours they averaged only 4.1 per cent. Whatever the true figure, the important point is that removal of the main liver, which can be accomplished by operation, entails a reduction in the amount of hepatic tissue well below that obtaining in most instances after a prolonged fast.

Removal of the Main Liver.

Under ether anesthesia, with aseptic conditions, and the rat on an electrically heated pad, the abdominal wall is slit through from the junction of the ensiform cartilage and manubrium to well below the anterior border of the liver, a distance of 3 to 5 cm. The exposed ensiform springs outward of itself, yielding access to the liver high on the diaphragmatic surface; and the incision is held open with an eye speculum. The filmy, tough membrane which loosely connects the under surface of the main liver with the papillary lobe is torn through, a small sponge is so placed as to hold the two widely apart, and a thin edged, flat, aneurism needle is gently thrust between the portal trunk to the main liver and the underlying vena cava. Several attempts will often have to be made before it passes. The eye of the needle is now threaded with very fine silk (No. 1 surgical) and the portal trunk with the accompanying artery and bile duct are all included in one ligature.

The remaining steps in the removal of the main liver are simple. The suspensory ligaments are torn through, freeing it save for the caval and gastrohepatic connections; a thread of strong, thin silk (No. 2 surgical) is carried around the mass between it and the diaphragm; the gastrohepatic omentum is picked up with curved mosquito forceps just above the portal tie and severed with scissors; and by the pull of the forceps, the mass is everted while the thread already in place at the junction of the hepatic and caval veins is tied down. If slight traction be made upon the thread as the knot is laid, it will come to include only the veins first mentioned. The liver mass can now be cut away at leisure, leaving a neat small stump. Closure of the abdominal wall is done in two layers, with No. 1 silk; and no dressing is applied. Healing by first intention is the rule. The whole operation can ordinarily be completed in 12 to 15 minutes. Passive congestion does not follow; and except for intercurrent disease there would be almost no mortality. About one animal in ten is lost from this latter cause.

The control rats were treated in every way like the ablation animals except that a laparotomy only was performed on them and the opening held apart with a speculum during the time ordinarily required for removal of the main liver.

Changes after the Ablation in Fed Rats.

In well fed rats the lobe mass increases in size with astonishing rapidity after removal of the main liver. Ponfick's[10] classical description of the course of events in rabbits so treated renders super-

[10] Ponfick, E., *Virchows Arch. path. Anat.*, 1895, cxxxviii, suppl. 81.

fluous a detailed account of the changes. The increase in tissue comes about by an interposition, to use Ponfick's term, of new cells here and there throughout the lobuli already existing. We find that in rats still young but sexually mature and weighing 130 to 150 gm., the lobe mass reaches the size of the entire original liver in from 9 to 12 days; whereas in old animals of 300 gm. or more it has attained only two-thirds this bulk after the longer of the periods mentioned. Many observations would be required for exact knowledge of the rate of regeneration at different ages, and fortunately it is not essential for present purposes.

Changes in Fasting Animals.

The effects of fasting were observed in six experiments, each with appropriate controls. Forty-five animals in all were successfully studied. In the early work the animals fasted for only 6 hours prior to removal of the main liver, and for this reason the influence of residual food in the intestine cannot be wholly ruled out, as it was later by a preliminary fast of 24 to 48 hours. Furthermore, the controls were not operated upon. The animals of a first series were kept on shavings, some of which they ate and their feces as well, while in another they were confined in metal metabolism cages and lost weight with great rapidity. But the results of these imperfect experiments were identical with those of the better ones and possess a corroboratory value.

Profound differences were regularly to be noted between the liver changes in the ablation rats and those in the controls killed at the same stage of advanced inanition. In the latter the atrophy of the entire liver, described by all previous observers, was well marked, whereas in the former the fragment of hepatic tissue left behind at operation had in most cases undergone an indubitable hypertrophy.

According to an extensive literature[11] the liver of inanition is remarkably small and flabby, and sharp edged, tough because of the great relative diminution in parenchyma, and suffused with blood owing to a widening of the intralobular capillaries consequent on

[11] Aschoff, L., *Path. Anat.*, 1911, i, 41. Morpurgo, B., *Beitr. path. Anat.*, 1889, iv, 313. Mühlmann, M., *Centr. allg. Path. u. path. Anat.*, 1899, x, 160.

atrophy of the liver cords (Fig. 3). Intralobular hemorrhages are said to occur but these we have not seen. All of the parenchymal elements do not suffer equally, the changes being much more marked toward the center of the lobule, the cell cords tapering from a relatively broad base of approximately normal elements next the interlobular connective tissue to a narrow acuminate column of tiny entities having a mere rim of protoplasm about a much diminished dark nucleus. The average diameter of the cells of entire lobules may come to little more than half the normal, while that of the nucleus may be about three-tenths of it,[12] figures which imply extreme changes in the elements near the central veins. Here the widening of the capillaries gives the picture a superficial similarity to that of chronic passive congestion. When the vessels are cut and the blood escapes the liver falls in on itself, so to speak.

All these changes were present in our control rats killed after 9 to 11 days of fasting, when 25 to 40 per cent of the body weight had been lost. But diametrically different, as already stated, were the findings in the ablation animals, although in them the loss of body weight was on the whole more pronounced as would follow from the mechanical removal of several grams of hepatic tissue, part of which might have been utilized secondarily, as food. In these emaciated creatures the small liver mass left behind at operation was in most instances enlarged and rounded, friable because of a disproportionate increase in its parenchyma, and, when the vessels were severed, rendered almost bloodless (Fig. 2) by the pressure of the growing tissue upon the capillaries. Microscopically, the changes were those of a genuine hypertrophy in Ponfick's sense, and similar in all ways to those occurring after a like ablation in well fed animals except that they were less marked. The individual lobuli were far larger than those in the main livers removed at operation from the same rats, owing to the increased length of the cell cords, which latter contained many more cells than is normal and these undiminished in size (Figs. 1 and 2). Globules of fat were not infrequently present in the parenchyma near the central vein, as in some well fed animals, but usually they were absent and the hepatic tissue appeared normal save for the changes incident to its proliferation.

[12] Lasarew, N., *Russ. Arch. Path.*, 1897, iii, 399, cited by Mühlmann, M., *Centr. allg. Path. u. path. Anat.*, 1899, x, 160.

The Influence of Age.

Jackson[13] has compared the effects of inanition on adult and young rats, and has pointed out that in the latter a growth impulse must be recognized as influencing the size of the liver. Our animals were well beyond the age at which this impulse becomes negligible, but, owing to the unusual conditions introduced by the ablation, experiments seemed called for to determine whether the hypertrophy of the lobe mass during inanition would take place in old animals. It does take place. In two old rats of 290 and 320 gm., subjected to an ablation, the same changes occurred as in ordinary adults (Figs. 1 and 2).

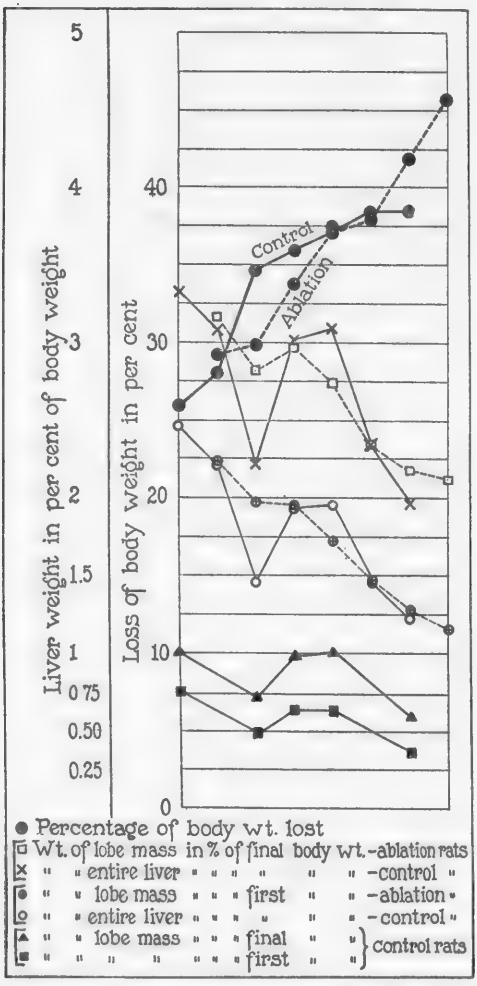
The Ultimate Amount of Liver.

As data accumulated, a significant fact made itself evident. Not only did the lobe mass hypertrophy in the ablation rats but its ultimate weight closely approximated that reached by the entire liver of the controls through a process of atrophy. The final experiments were framed with special attention to this point. In order to rule out completely the factor of residual food as a stimulus to hypertrophy the animals were subjected to a preliminary fast of from 24 to 48 hours in jars with a dais. So rapid are body processes in the rat that the main livers removed after this brief period showed plainly the changes characteristic of early inanition (Fig. 1).

The results of one experiment will be given *in extenso* (Table I, Text-fig. 1). The data from all the closely controlled material find expression in Text-fig. 2.

Experiment 6.—Fifteen well nourished male adult rats, closely paired in weight and length, were operated upon after a 24 hour fast. Their weight just prior to anesthesia is the "first weight" of Table I. The main livers were removed from seven of them; while in the case of the others a laparotomy only was performed. Despite continued fasting, all made a good recovery. One ablation rat (No. 15) died of inanition 7 days after the operation,—or after 8 days in all, without food. It was autopsied practically at once. The findings offer an extreme example of the changes manifest in the others. These were killed on the same day while still in good condition. The abdominal wounds were healing with extreme slowness though by first intention.

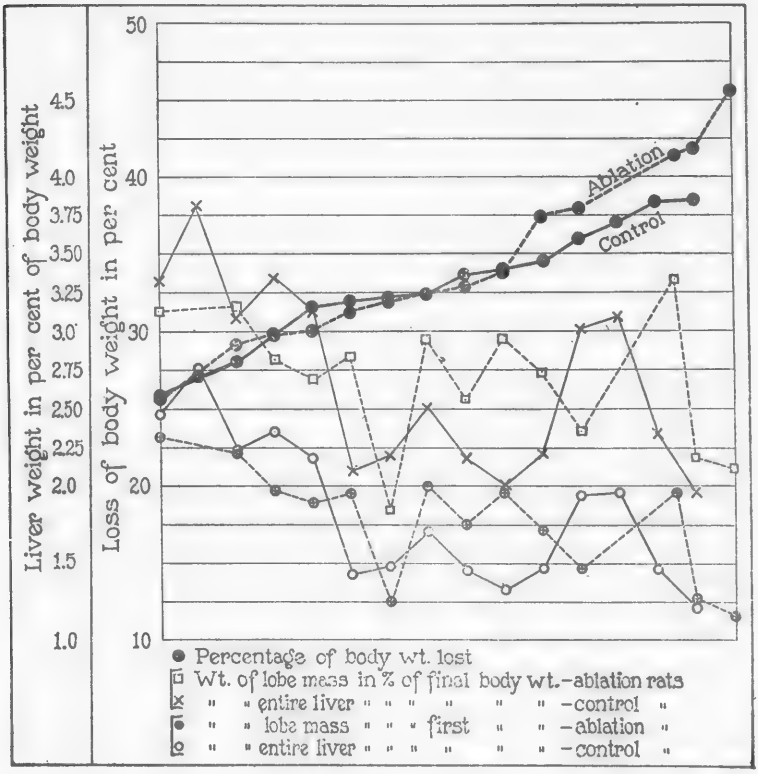
[13] Jackson, C. M., *J. Exp. Zool.*, 1915, xix, 99.


Liver weight in per cent of body weight
5
4
3
2
1.5
1
0.75
0.50
0.25
Loss of body weight in per cent
40
30
20
10
0
Control
Ablation
● Percentage of body wt. lost
□ Wt. of lobe mass in % of final body wt. - ablation rats
× " " entire liver " " " " " " - control "
● " " lobe mass " " " first " " - ablation "
○ " " entire liver " " " " " " - control "
▲ " " lobe mass " " " final " " } control rats
■ " " " " " " first " "

Each solid black dot records the loss in body weight of a rat. On the same perpendicular there are placed other symbols recording data on the liver weight of the animal, as expressed in terms of the body weight. In order to distinguish between the material of the control and ablation rats, the symbols pertaining to each series have been joined up with lines.

TEXT-FIG. 1. The duplication occurring in the liver weights of fasting ablation animals and controls (Experiment 1).

According to Jackson, white rats may be expected to tolerate an average loss through fasting of about 40 per cent of the gross body weight. The animals of the present experiment lost between 25.8 and 45.7 per cent of an approximately *net* body weight, which is to



TEXT-FIG. 2. The duplication occurring in the liver weights of fasting ablation animals and controls (general results).

say that some of them were at the extreme of inanition. Those of the ablation series were in general most affected as in the previous series. To cope with the difference thus introduced into the data these latter have been grouped in the chart with special reference to

the body loss, the findings in each ablation animal being superimposed upon those of a control that had lost to the same amount. But since the nutritive condition of the animals at the end of the experiment was not always accurately expressed by the percentage loss in tissue, descriptive terms have been employed as well in the table. In the "extremely thin" animals only traces of fat could be seen anywhere in the gross at autopsy; in those that were "very thin" there still existed a little fat in the omentum and retroperitoneal tissue but none in the subcutaneous tissue; while in the "thin" creatures there was some fat at this latter situation and more within the peritoneal cavity. The one rat that was merely "rather thin" had still much intraabdominal fat.

In the analysis of the findings one control (No. 8) was ruled out because of the "giant spleen" with which an abnormally large liver is associated. The animal had lost more weight (39.4 per cent) than any other, yet the liver had undergone almost none of the usual atrophy and histologically the tissue appeared normal. The instance well illustrates the importance of the hepatolienal complex as affecting work with the rat liver.

For purposes of comparison the weights of the entire liver of the controls and of the lobe mass of the ablation series have been expressed in percentages both of the initial body weight and of that at the time of death. No inconsiderable proportion of the bulk of the atrophic control livers was made up of blood contained in the sinuses between the atrophic parenchymal columns, whereas in the hypertrophic lobe masses of the experimental animals this element was almost lacking, as already mentioned.

The liver of one of the control rats losing greatly (No. 7) had so far atrophied that its final weight was, on calculation, about the same as that of the lobe mass alone at the time of operation. It now constituted 1.21 per cent of the initial body weight, whereas the lobe mass at the beginning may be supposed to have come to about 1.28 per cent, allowing that this mass constituted 31.2 per cent of the tissue of the original whole liver and that the latter amounted to 4.1 per cent of the body total. In two other cases (Nos. 3 and 6), there was almost as great a degree of atrophy. The actual diminution in parenchyma was even greater than the figures indicate because of the suffusion of the atrophic tissue with blood.

In the ablation animals corresponding with the more extreme of these instances, there was no need of hypertrophy of the lobe mass for it to be as large as the total liver of the controls. The mass need only hold to its original bulk, or, when this was exceptionally great, it might even atrophy a bit. And this was precisely what happened. In ablation Rat 14 there occurred, as the figures show, what may be termed a permissible slight atrophy, while in No. 13 the tissue did not alter from the normal. These cases stand in an enlightening contrast to others in which an amount of liver tissue equivalent to that present in the controls came into being through an active hypertrophy. In three ablation rats the bulk of the lobe mass must have practically doubled during the short period after the operation. Its final weight equalled that of the main liver that had been removed from the same individuals, and was several times that of the lobe mass in the controls, which was extremely small as the chart shows.

Nature of the Changes.

The first question in connection with these findings is whether they may not be accidental. The enlargement of the lobe mass following the ablation might perhaps be set down as inevitable to the increased flow of portal blood, did not atrophy or no change at all sometimes occur instead. In many species there takes place early in starvation an active transfer of body fat to the hepatic cells which show large or small globules of it, especially at the periphery of the lobuli. In the rat, according to Mottram[14] this transfer is lacking, at least to any noteworthy extent; and under the conditions of extreme inanition with which we are here concerned, it is negligible even in the species mentioned. In our rat livers, fat was visible only in the largest of the hypertrophied lobe masses wherein some globules were to be seen. These made up but a negligible portion of the total bulk and were central in situation instead of peripheral, as are those of fat transferred ordinarily during a fast.[15]

Analyses have shown that the liver tissue in instances of death from inanition yields about 10 per cent of fat, which is not demon-

[14] Mottram, V. H., *J. Physiol.*, 1909, xxxviii, 281.

[15] Statkewitsch, P., *Arch. exp. Path.*, 1894, xxxiii, 415.

strable histologically.[16] The atrophic and hypertrophic tissues of the present experiments might well be found to differ in such respect. Yet assuming this to be the case, one still could not be certain whether it was not perhaps the expression of a difference in the essential make-up of the liver protoplasm in the contrasted series of animals, rather than one in amount of extraneous material there deposited. That there was a difference in the amount of parenchymal protoplasm present in a like weight of the atropic and hypertrophic liver tissue is certain. The proportion was less in the former, owing to the suffusion of it with blood. Furthermore, the quantitative relationships between cytoplasm and nucleus were markedly abnormal in the atrophic control livers, as others before us have noted,[12] whereas there was in this respect but little that was unusual in the cells of the hypertrophic lobe masses. If it is impossible to state of two like bulks of cells of the same appearance and apparent condition that they have identical functional capacities, how much more vain would it be to assert this of unlike bulks with cells similar only in their derivation as in the present case!

The fact remains, and remains significant, that there was a close approximation in weight between the liver masses in our two sets of animals and that this occurred, in the one case by a simple atrophy, in the other by what may be termed with equal reason simple hypertrophy. And not only this, but when an hypertrophy of the lobe mass was rendered unnecessary by the circumstances of the case, atrophy of it or no change at all was what occurred, with result that the mass still duplicated in weight the main liver of the appropriate control. This is strong evidence that both bulks of tissue, whatever their incidental difference in parenchymal content and in appearance of the cells, constitute expressions of a response to body demands of like magnitude. One may conceive of both parenchymas, though so different histologically, as having to perform and performing the same amount of work.

Significance of the Findings.

There are precedents in the behavior of other organs for the hyper-

[16] Rosenfeld, G., *Ergebn. Physiol., 1. abt.*, 1903, ii, 50, 86.

trophy of the liver that occurs during starvation. McCarrison[17] has shown that in fasting pigeons the adrenal glands enlarge, and Jackson's figures indicate that they do so in rats that are fasted. Very recently Vincent and Hollenberg[18] have presented data which show that in this latter species a nearly fourfold increase in the weight of the adrenals takes place during an inanition period of 10 to 13 days, and an almost threefold increase in the thyroid weight, which is accompanied by a disappearance of the colloid material. These findings suffice to show that the liver changes are not unique in character.

Mention has already been made of the alternative explanations which have been advanced to account for the pronounced hepatic atrophy in fasting individuals. According to one set of workers the organ is primarily used up as food, while others hold that it decreases in amount because of the decrease in functional demands. Needless to say, the results of our experiments are wholly in keeping with this latter view. The explanation that the liver tissue necessarily undergoes destruction for food is rendered untenable not alone by our data but by the demonstrated hypertrophy of certain other organs during inanition, in contrast to the atrophy then generally occurring. That there exists a "padding" of glycogen and fat in the liver, which is utilized as food early in inanition no one will deny, nor that somewhat later, fat mobilized from elsewhere in the body may be deposited in the organ only to be removed again; while that liver tissue disappearing at late stages in inanition is secondarily utilized as food can scarcely be doubted. In this connection it may be remembered that our control animals emaciated less rapidly than those from which liver tissue had been removed (Table I). Yet with all said, there is excellent reason to conclude that the primary cause for the disappearance of liver tissue during inanition lies in a lessened functional need.

Extent of the Changes.

In discussing the extent of the liver changes we shall make use of the data of Experiment 6 only, inasmuch as this experiment was the most carefully carried out and controlled. The diminution in hepatic

[17] McCarrison, R., *Indian J. Med. Research*, 1918–19, vi, 275.

[18] Vincent, S., and Hollenberg, M. S., *J. Physiol.*, 1920–21, liv, p. lxix.

tissue in our control animals amounted on the average to 53.7 per cent. In Jackson's rats with inanition comparable in degree it came to 58 per cent, but his normal average for the liver, which formed the basis of calculation, was higher than ours, owing to the circumstance that it had been obtained by observations on the organs of full fed rats instead of ones subjected as ours were to a brief fast. The amount of liver tissue ultimately present in our rats varied with the loss of body weight. It ranged in the case of the controls from 1.21 to 2.46 per cent of the initial body weight, with an average of 1.81 per cent instead of the 4.1 per cent that is normal. In the experimental animals the ultimate amount of liver tissue was smaller, from 1.15 to 2.24 per cent of the initial body weight, with an average of 1.68 per cent. This is below the average for the controls but the animals were in general thinner. The chart demonstrates that under like individual conditions the liver weight was the same for both series.

The Liver Requirement During Inanition.

If it be assumed, as the facts seem to warrant, that the liver weight late in inanition is determined by the functional needs, then the data of our experiment should give some indication of the dimension of these needs. The normal liver makes up 4.1 per cent of the net body weight. But of this weight some 10 per cent consists of what Jackson[6] terms the ligamentous skeleton, structures which alter but little in the adult even during inanition and which must make an almost negligible day-to-day demand on the hepatic tissue. The normal percentage relationship of the liver weight to the net body weight minus that of this skeleton, that is to say the relation of the liver weight to the soft body weight comes to 4.56 per cent. In our animals the final liver weight as expressed in that of the final body weight was for the controls 2.71 per cent and for the operated rats 2.61 per cent—the general average being 2.66 per cent. But according to Jackson, the ligamentous skeleton makes up some 15 per cent of the final body weight. When allowance has been made for this the liver percentage as expressed in the weight of the soft body comes to 3.2 per cent, or to about three-fourths of the normal. Of the missing

quarter some part assuredly consisted of "padding,"—fat and glycogen on temporary deposit in the organ. Altogether the findings indicate that during inanition there is large need for a liver.

Possible Nature of the Requirement.

What is the nature of this need? The hepatic tissue is known to be busily concerned under normal conditions with the tasks of intermediate metabolism. During inanition the intermediate metabolism still goes on, though it is true that the substances worked upon are no longer derived from the digestive tract but mobilized from the tissues.[19] Some of them, fat and to a less extent glycogen,[20] are temporarily laid down in the hepatic cells. In the case of fat, there may be wave-like fluctuations in the amount present from day to day, as inanition progresses.[14] The fact that under circumstances which involve a ruling out of ordinary digestive processes the liver still remains large, relatively speaking, does not necessarily mean that it is not normally a portal organ, receiving on the blood from the digestive tract the functional impacts, so to speak, which are responsible for its size. For impacts of the same sort still come to it during inanition but from a different source and direction.

After an Eck fistula has been produced the liver undergoes a permanent reduction in size of about one-fifth.[21] If functional demands determine liver size this can only mean that the organ has less to do. For the alternative explanation, that enough blood cannot be supplied through the hepatic artery to nourish a larger organ, is untenable. There are too many instances in which, on occasions demanding functional hypertrophy, the blood supply undergoes an increase *pari passu* with the tissue increase, as *e.g.*, during the compensatory adjustment after removal of a kidney. In the case of the Eck fistula liver, one may suppose that some share of the normal hepatic task is, owing to the changed circulatory arrangement, performed elsewhere in the body. For many of the normal activities of the liver are not unique with the organ but are shared by others to a less extent.

[19] Lusk, G., Science of nutrition, Philadelphia, 3rd edition, 1917.

[20] Junkersdorf, P., *Arch. ges. Physiol.*, 1921, clxxxvi, 238. Laquer, F., *Klin. Woch.*, 1922, i, 822.

[21] Whipple, G. H., and Hooper, C. W., *Am. J. Physiol.*, 1916–17, xlii, 544.

During inanition this is doubtless still the case. And while the portal flow still goes on at such a time, the portal blood cannot but differ much less from the systemic than usual. It no longer carries with it so many special functional opportunities. The failure of the liver of fasting animals to be quite as large, relatively speaking, as the ordinary, may be consequent on this changed condition of affairs, whereby the liver loses in opportunity over the other tissues.

The character of the demands made upon the liver cannot but differ much in early and late inanition. So long as the fat reserves of the body hold out, the metabolic needs are supplied almost entirely from this source.[19] It is unnecessary to refer again to the evidence of fat mobilization and transport to the liver. Later when the body fat is exhausted, protein is called upon, and now emaciation proceeds with a special rapidity owing to the amount of tissue used up for the needs of each day. In our ablation animals it was noteworthy that whereas those killed while still in possession of some fat, though much emaciated, had a hypertrophic lobe mass, others fasting only for the same time but with a practically complete exhaustion of the fat deposit possessed an hepatic tissue that was either atrophic or unchanged. These latter had begun to use up tissue protein at an unprecedented rate; yet the livers were small. The question arises whether this was because the demands on the organ were less when protein was burnt or because the needs of the body for food took precedence over those for a large liver. Certain facts indicate that the explanation first mentioned may be the correct one. The body is able to handle proteins elsewhere than in the liver. Atrophy is not inevitable to extreme inanition, as the hypertrophy of the adrenal and thyroid sufficiently shows. While at the time when death occurs from inanition abundant food for the cells may still be present in the body.

SUMMARY.

In rats fasted after the removal of about seven-tenths of the liver the remaining fragment undergoes ordinarily a marked simple hypertrophy and attains the weight reached through a process of simple atrophy by the entire liver of fasting controls. Under circumstances of exceptionally severe inanition, the hypertrophy may not occur, the

fragment remaining unchanged or even undergoing a slight atrophy. But since in comparable controls the hepatic atrophy is extreme, the duplication in the liver weights still manifests itself.

It is difficult to avoid the conclusion that the liver changes which take place in fasting animal are essentially conditioned on functional demands made upon the organ. Yet great as is the hepatic atrophy then occurring, this does not necessarily mean that these demands are much lessened during inanition. For a comparison of the liver weight with that of the soft body (net body weight less the weight of the ligamentous skeleton) brings out the fact that the organ is, relatively speaking, three-fourths as large at extreme inanition as in the well nourished individual. Of the missing fourth a part may be foodstuffs on temporary deposit. And the rest is perhaps to be accounted for by the existence of special conditions during inanition which favor the vicarious assumption of a part of the usual liver work by the other tissues.

While the findings show that the liver size is essentially dependent on functional demands, they do not enable one to say whether the special demands in question come normally to the organ by way of the portal stream.

EXPLANATION OF PLATE 21.

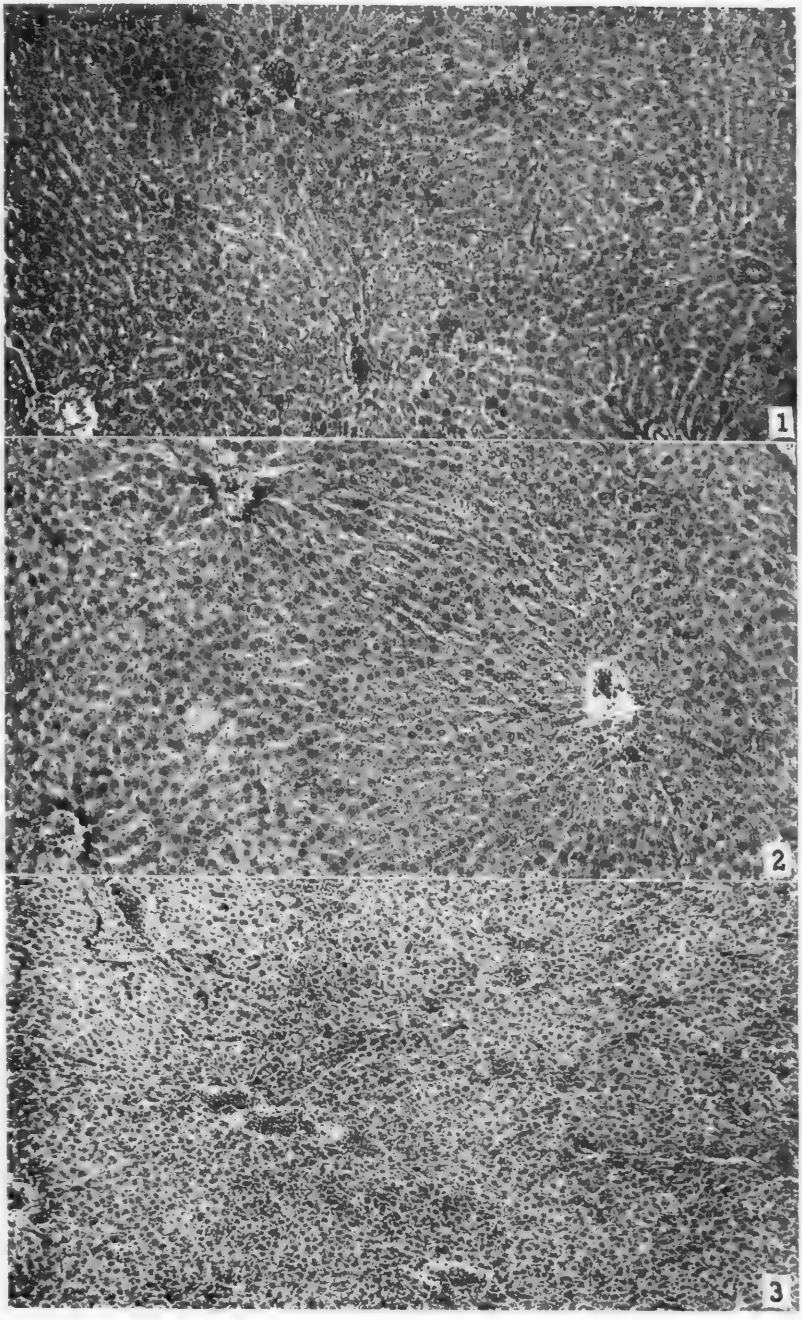
FIGS. 1, 2, and 3. To show the histological character of the liver changes in an ablation rat and its appropriate control. All of the figures have the same magnification, 150 diameters. Eosin and methylene blue.

FIG. 1. Section of the main liver removed after a 48 hour fast from a rat of 290 gm.

FIG. 2. Section of the lobe mass of the same animal after 7 days more without food.

FIG. 3. Section of the lobe mass of the fasting control animal which was submitted merely to a laparotomy. The initial weight of this rat was 302 gm.

The control lost 36.7 per cent in body weight and the ablation rat 37.4 per cent. The lobe mass of the latter weighed 4.64 gm. at autopsy, whereas the entire liver of the control came to only 4.78 gm.



(Rous and McMaster: Fasting organism.)

UNIV OF MICH.

INDICATIONS OF TISSUE SPECIFICITY IN A TRANSPLANTABLE SARCOMA.

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(Received for publication, June 25, 1923.)

That certain aspects of susceptibility to transplantable tumors are genetic phenomena has been demonstrated in the case of a carcinoma and a sarcoma of the Japanese Waltzing mice[1,2,3] and in two mammary adenocarcinomata occurring in a special strain of highly inbred common domestic mice.[4,5] The acceptance of the view that susceptibility to transplanted neoplasms is fundamentally a genetic problem encounters an obstacle in those transplantable tumors which grow in all inoculated individuals, irrespective of the strain of mice employed. This is particularly true of a sarcoma transplanted at the George Crocker Research Laboratory Fund, Columbia University, and designated as No. 180, a tumor which grows almost uniformly in a number of different strains of laboratory mice from several sources.

The following experiments have been carried out, not so much for the purpose of discovering a race resistant to this tumor, although that was the original intention, as to determine whether there are any indications of genetic phenomena associated with its transplantability. About 400 mice have been employed, a number insufficient for conclusive data, but as the work has been unavoidably interrupted, it seems advisable to present the data as they stand at present.

[1] Little, C. C., and Tyzzer, E. E., *J. Med. Research*, 1915–16, xxxiii, 393.

[2] Little, C. C., *Science*, 1914, xl, 904.

[3] Tyzzer, E. E., and Little, C. C., *J. Cancer Research*, 1916, i, 387.

[4] Strong, L. C., and Little, C. C., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 45.

[5] Strong, L. C., *J. Exp. Zool.*, 1922, xxxvi, 67.

Methods.

The usual methods of transplanting by means of a trocar were used in these experiments, care being taken to get uniform sized grafts. Individuals belonging to the several contrasted groups of mice were inoculated with tissue from the same tumor mass on the same day. In some experiments individuals of a particular group were inoculated immediately preceding individuals of a contrasted group; in the next experiment the order of inoculation of the two groups was reversed.

We have attempted to work out a technique for determining the growth rate of the transplanted sarcoma but any estimate of this kind, especially for the tumor used in these experiments, is subject to many errors caused by hemorrhages into the tumor, areas of necrosis, etc. Causes for individual variations in growth rate are still obscure but factors known to influence this are intercurrent disease, local infections, and lack of uniformity in the stock of mice employed. In the present account we have not given the individual growth rates but rather the averages for the number of animals in the various groups. The method used was as follows: A number of mice were inoculated with the tumor and 1 week later the tumors were measured and recorded as accurately as possible; then some of the mice were killed and the tumor masses weighed to a tenth of a gram and the ratio between the measurement and the weight worked out. This procedure was repeated weekly up to the 4th week after inoculation and in some cases, to the 5th, but it was not considered worth while to continue longer as the tumor became soft, and extensive necrosis was common after this period.

The individual growth rates in the experiments were determined either by comparing the measurements with the weights of the standard series, or by taking the actual weight of the tumors at the end of the experiment. The former method is crude but it, in all probability, gives an approximation to the true weight. The method of the estimation of the weight of the tumor mass was used only for the first 2 weeks of the experiments. The data for the 3rd, 4th, and 5th weeks were based upon actual weights. The degree of significance between two contrasted groups of individuals, being based primarily upon the data of the latter part of the curves, is therefore valid.

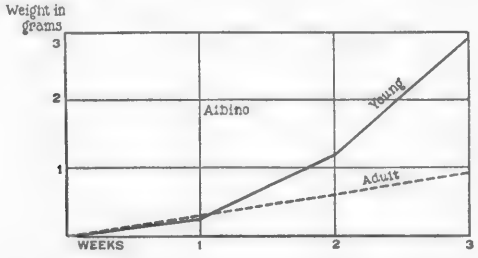
EXPERIMENTS.

Experiment 1.—For the first experiment the behavior of the sarcoma was studied in the special strain of albino mice,[4,5] a stock rendered relatively homogeneous by the mating of brother to sister over a period of several years. Two classes of animals were used: (1) sexually immature mice averaging about 6 weeks old, the limit being 5 to 7 weeks, and (2) adult mice, over 2½ months old and apparently sexually mature. The growth rates of the tumor in these two groups are given in Table I and Text-fig. 1.

TABLE I.

Average Growth Rate of Tumor in Albino Strain.

Wks.....	1	2	3
	gm.	*gm.*	*gm.*
20 young.....	0.22	1.15	2.86
19 adults.....	0.24	0.57	0.91



TEXT-FIG. 1. Average growth rate of tumor in albino strain.

While this sarcoma grew progressively in all of the mice inoculated it is evident from the figures that the rate of growth was relatively faster in the immature than in the mature individuals.

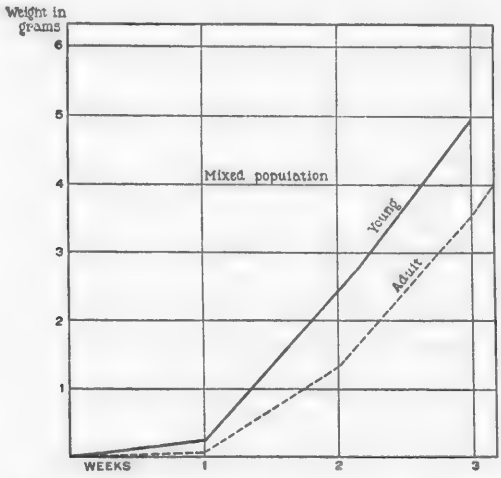
Experiment 2.—The mice selected for inoculation in this experiment were of a relatively heterogeneous strain as compared to the homogeneous stock of the first experiment. The individuals were descendants from a cross between two inbred strains or else mice produced by the promiscuous mating of a number of domesticated mice from several sources. This has been brought about by including only those mice of known hybrid constitution. By the process of outbreeding, that is by the mating together of mice that are known not to be closely related, the degree of heterogeneity, or hybridity, of the offspring was increased. Several distinct stocks of mice were used for the parent generations but it should be clear

that the mice forming the parent stock were not included in this experiment since most of them were from inbred and relatively homogeneous stocks. These parent stocks were (1) the inbred albino strain used in Experiment 1, (2) a highly inbred stock known as the "dilute browns," and (3) a stock of partially inbred yellow mice. In addition another group of mice known as market mice, including individuals from several distinct sources, was also used for inoculation with the sarcoma. A deliberate attempt was made to render this group as heterogeneous as possible. The results obtained from inoculating the sarcoma in this stock, divided into immature and mature individuals, is shown in Table II and Text-fig. 2.

TABLE II.

Average Growth Rate of Tumor in Mixed Strain.

Wks.....	1	2	2+1 day.	3	3+1 day.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
79 young.....	0.24	2.40	2.70	4.90	
203 adults.....	0.07	1.25		3.50	3.90



TEXT-FIG. 2. Average growth rate of tumor in mixed population strain.

The results from inoculation in the heterogeneous strain show, as in the first experiment, a more rapid growth of the tumor in the immature mice.

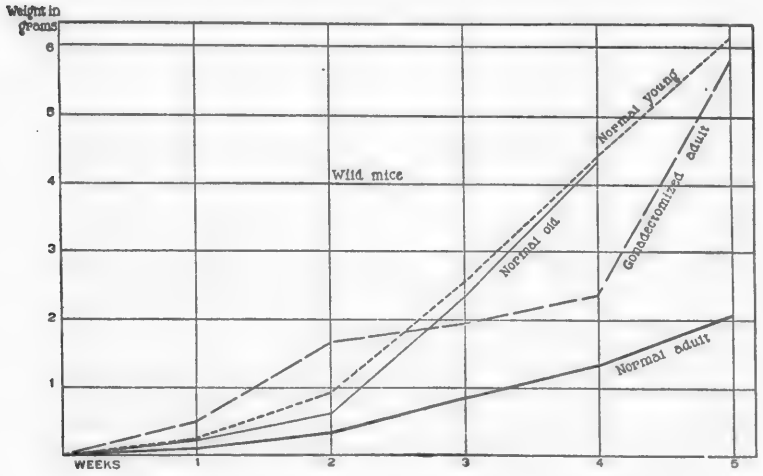
Experiment 3.—A strain of wild mice which had been caught in the pigeon coops of the Carnegie Institution Laboratory at Cold Spring Harbor were used

for this experiment. Some of the individuals had been inoculated a year or more previously with an adenocarcinoma but on account of the lapse of time and the fact that none of them had shown more than a transitory growth, it was considered safe to use them for the present experiment. This wild strain was divided into three groups: (1) young, between 4 to 9 weeks old, (2) adults, above 3 months and sexually mature, and (3) old, individuals apparently past sexual activity.

TABLE III.

Average Growth Rate of Tumor in Wild Strain.

Wks.	1	2	3	4	5	6
	gm.	gm.	gm.	gm.	gm.	gm.
21 young	0.22	0.97	2.55	4.40	6.10	2.423
14 old	0.22	0.64	2.35	4.36		
24 adults	0.15	0.33	0.89	1.35	2.08	0.960
8 castrated adults	0.52	1.68	1.84	2.37	5.76	2.434



TEXT-FIG. 3. Average growth rate of tumor in wild strain.

The individuals that were grouped together as old mice were those that had been classified as adults of 10 to 14 months of age, a year previously. In addition it is possible to recognize old age in wild mice fairly definitely. The relative size, sparsity of hair, especially on the head, decreased activity, and pugnacity, especially among the males, are all so well marked that classification is not difficult. The results of inoculation of these groups are shown in Table III and Text-fig. 3.

The experiment evidences again the greater susceptibility of the immature animal over the normal adult and furthermore it adds the surprising fact that the mice past sexual activity have a susceptibility equally as great as that of the immature individuals.

Experiment 4.—Some of the animals from the wild strain had been castrated 10 and, in some cases, 12 months previous to the present experiment. There were two groups of these, one in which the animals had been castrated before sexual maturity, and the other group castrated during sexual maturity.

The nine mice of the first group showed complete resistance to inoculations of the sarcoma, while twenty-one controls of the same

TABLE IV.

Summary of Percentage Indications of Progressive Growth for All Classes of Mice Employed.

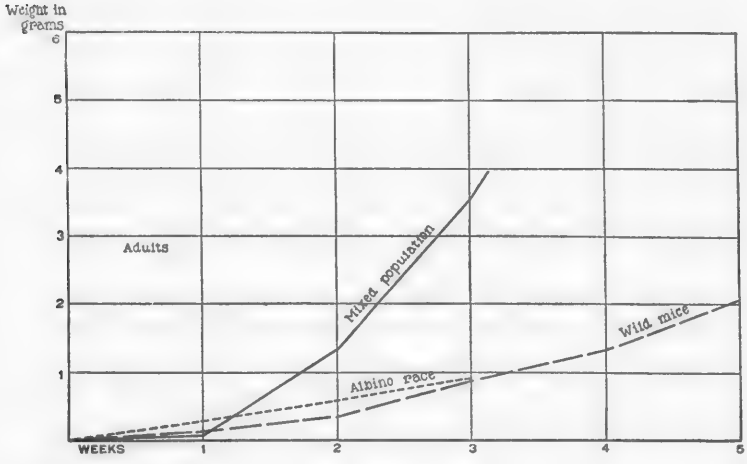
Mice.	Age group.	Strain.	No. susceptible.	No. resistant.	Percentage susceptible.
Normal.	Adult.	Mixed.	203 ± 1.15	3	98.5 ± 0.5
"	Young.	"	79	0	100
"	Adult.	Albino.	19	0	100
"	Young.	"	20	0	100
"	Adult.	Wild.	24 ± 1.5	6	80 ± 5
	Old.	"	14	0	100
	Young.	"	21	0	100
Castrated.	Adult.	"	8	0	100
	Young.	"	0	9	0.0

age and stock were uniformly susceptible. In the second group, castrated a year previously but by an operation performed during sexual maturity, all individuals grew the sarcoma progressively and at a rate greater than that found in normal individuals of the same class and equalled only by the rate in immature and old mice of the previous experiment (Text-fig. 3). The control mice from the same class had a susceptibility of 80 per cent ± 5. It is possible that these gonadectomized adult mice had gone over into the old age stage which would explain the increased growth rate of the tumor.

Whether the differences are significant is problematic since the number of mice employed in the gonadectomy experiment is small. However, resistant animals among the normals are so extremely

rare, that it seems to us that the results in the first group, operated on before maturity with complete resistance in nine mice to the tumor inoculation, is suggestive even with so small a number of animals.

For comparison the percentage of takes in all of the above experiments is given in Table IV and the growth rate for the tumor in adults of the three strains of mice employed in Text-fig. 4.



TEXT-FIG. 4. Average growth rate of tumor in adults of the three strains of mice employed.

DISCUSSION.

The fact that the physiological changes within an organism, due to age, have a significant influence upon a host's tolerance of a transplanted tissue has been fairly well established.[6] Very young individuals of a non-susceptible race will sometimes grow a transplantable tumor, although no adult individual of the same race will grow the same tissue; *vice versa*, immature individuals of a susceptible race will sometimes fail to grow a transplantable neoplasm, yet the same individuals will grow the same tissue when they become sexually

[6] Little, C. C., *J. Exp. Zool.*, 1920, xxxi, 307.

mature. Old mice beyond the stage of sexual activity react to transplantable neoplasms in a way similar to immature ones.[5]

In the case of the particular sarcoma used in these experiments, it was impossible to determine any indication of tissue specificity by the study of the percentage of susceptible mice within any particular age group since there was practically 100 per cent susceptibility for all classes of normal individuals except in the case of wild mice in which there was approximately 80 per cent susceptibility. This sarcoma is evidently of such a nature that it calls forth no resistance from normal individuals of the several available stocks of mice. This fact is not contradictory to a genetic interpretation of susceptibility to transplantable tumors, as all our domestic breeds of mice have possibly had a common origin, so that it is more than probable that all breeds have at least some genetic factors in common. Genetic factors are involved for susceptibility to certain neoplastic tissues and should not be excluded in those cases in which all individuals grow the transplanted tissue.

The fact that adult mice grow the sarcoma more slowly than do immature ones would indicate that during the stages of sexual development there is a gradual increase in the vital resistance of the host to the transplanted tissue. In other words, the host possesses the phenomenon of tissue specificity to some extent, at least, to this particular neoplasm. Old mice revert to conditions analogous to those of the immature stages in as far as both classes give the same growth rate curve, although the conditions that bring about this result may not necessarily be the same. At any rate, there is an indication that with the onset of senescence there is possibly a concomitant loss of at least some of the tissue specificity.

The experiments dealing with gonadectomized individuals give more evidence for the assumption that the phenomenon of tissue specificity is accountable for differences in growth rates between the different age groups. Gonadectomy probably does not change the percentage of susceptible individuals, provided the individuals be mature when operated upon. This bears out a conclusion derived from the work on two adenocarcinomata reported in a previous investigation.[5] The only discernible effect of gonadectomy upon adult individuals is a slight increase in the growth rate of the transplanted

sarcoma. The conclusion seems warranted, therefore, that by the removal of the gonads from adult individuals one may remove certain checks that function against the transplanted tissue in normal individuals.

Another point concerning the work on gonadectomy needs to be discussed. If young, immature mice are operated upon, then kept a year, all of them fail to grow the sarcoma although 100 per cent of normal individuals of the same age class are susceptible. Apparently gonadectomy has had some determining influence upon the physiological mechanism that controls the transplantation of tissue. The conclusion to be drawn for this experiment is that the gonads have, by some means, a significant effect, to some extent at least, upon the assumption of tissue specificity within the organism. This is a verification of the same conclusion reached in the previous paper already referred to.[5] The numbers in this particular experiment are so small, however, that the conclusion can be only provisionally stated. That these results differ from those of other investigations upon gonadectomy may possibly be explained by the fact that the operated individuals were kept approximately a year before inoculation.

The last point deals with the growth rates for adult individuals from different sources. Mice from a homogeneous race grow the sarcoma much more slowly than do individuals from a heterogeneous or mixed population. Tyzzer and Little determined that F_1 hybrids between the susceptible Japanese Waltzing mice and a non-susceptible race grew a sarcoma faster than the original homogeneous Japanese Waltzers. The homogeneity and heterogeneity of organisms are, to a large degree, the manifestation of the genetic constitution of the individuals. The conclusion is warranted, therefore, that perhaps the growth rate of the sarcoma, used in the present experiment, is in some way correlated with the genetic constitution of hosts. If this conclusion be true then it is evident that we have a mechanism that will explain the variable growth rate of the neoplastic tissue in a group of market mice.

This sarcoma does, then, apparently have some characteristics that can be explained by the genetic theory of the transplantation

of tumors. There are other obscure matters concerning this tumor, of interest from the genetic standpoint, that can only be determined by future investigation.

CONCLUSIONS.

1. A sarcoma of the mouse which has proved to be transplantable to a great variety of strains of mice does not furnish an exception to the theory that transplantability is controlled by genetic factors, since this tumor possesses some characteristics that are more than probably genetic.

2. The phenomenon of the assumption of tissue specificity on the part of the host may possibly be of genetic origin.

3. The evidence suggests that the gonads have some influence on the assumption of tissue specificity on the part of the host.

4. The growth rate of the transplanted tumor may possibly be correlated with the genetic constitution of the host.

THE INFLUENCE OF THE ADRENAL GLANDS ON RESISTANCE.

II. The Toxic Effect of Killed Bacteria in Adrenalectomized Rats.

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(Received for publication, October 11, 1923.)

In another paper the remarkable effect of pyogenic infections on the adrenal cortex is reviewed (1). The hypertrophy of the adrenal cortex found in chronic infections may reasonably be interpreted as due to an increased demand made upon the physiological function of that tissue. The characteristic lipoids in the cells of the cortex which are present even in complete fasting rapidly disappear in severe pyogenic infections as shown both by experimental studies and by human autopsy findings. These pathological changes suggest a participation on the part of the adrenal glands in the resistance to infection.

Several investigators have found an increased susceptibility of adrenal-insufficient animals for certain drugs (Lewis (2, 3); Boinet (4); Schwarz (5); Giusti (6); Scott (7)). Also the susceptibility to diphtheria toxin is increased in adrenal insufficiency (Lusena (8); Frisco (9); Oppenheim and Loeper (10); Lewis (3)). Lusena showed that rats, though naturally immune to diphtheria toxin, were susceptible to it after adrenalectomy. In spite of this interesting alteration in the defensive mechanism to certain toxic substances the literature offers few data on the influence exerted by adrenal function on the resistance to pyogenic infections, the usual causes of adrenal change in human pathology. Several early observers reported a slight increase in the resistance of animals to bacterial intoxication[1] after the removal of one adrenal (Langlois and Charrin (11); Oppen-

* Arthur Tracy Cabot Fellow in charge of the Laboratory of Surgical Research of Harvard Medical School, 1922–23.

[1] Here and throughout this paper the term bacterial intoxication is used to designate the harmful effect of bacterial substances, and is not to be understood as limited to the effect of soluble toxins.

heim (12); Lucibelli (13)). Obviously unilateral adrenalectomy as a method for producing adrenal insufficiency is unsatisfactory.

The only direct investigation of the resistance to pyogenic bacterial intoxication in adrenal insufficiency that we have found in the literature is by Frisco (9). Two adrenalectomized rabbits were susceptible to a dose of *Staphylococcus aureus* culture which produced no obvious effect in controls. One of the former was killed in 12 hours and the other developed an abscess in the ear. This author also claimed that the blood serum of the adrenalectomized rabbits had lost much of its bacteriolytic activity.

It is the purpose of the present paper to report a study of the resistance of adrenalectomized rats to bacterial intoxications.[1] By this method we hope to throw some new light on the involvement of adrenal function in the pyogenic process. In this study killed bacteria were used throughout in order to separate the factors of intoxication from those of invasion. The resistance of adrenalectomized rats to living organisms is now being investigated.

Methods.

Procedure.—The experiments were conducted upon the following plan. Rats were observed for several days under standard conditions and then operated upon.[2] Both adrenals were removed at the same time to produce adrenal insufficiency. The operative technique employed and the methods of caring for adrenalectomized rats have been previously described (7). A certain number of each group of rats were used as controls, and for this purpose one adrenal was usually removed. Other operations such as thyroidectomy and splenectomy served as control procedures in other cases.

At varying periods after operation the resistance of the rats to bacterial intoxication was tested. Streptococcus and staphylococcus were used, since they are the most important organisms in human pyogenic infections. The animals were injected intraperitoneally with killed bacteria, either in a single injection of a heavy suspension or in smaller amounts repeated daily. The doubly adrenalectomized rats when tested in this way had all recovered from the operation, were eating well, and could not be distinguished from the normal and operative control rats in adjoining cages.

RESULTS.

1. Acute Intoxication.—A heavy suspension of streptococcus was used to produce acute intoxication.

[2] All operations were performed under ether anesthesia.

The organism used was isolated from a human empyema case at the Massachusetts General Hospital. It was not hemolytic after prolonged cultivation on artificial media. The organisms (24 hour growth on chocolate agar) were suspended in saline and killed by heat. Two lots of the bacterial suspension were required, each of about 250 cc.; they were kept on ice in tubes containing about 10 cc. each. No chemical preservative was used. The first lot contained 35 billion streptococci per cc., the second 31 billion per cc. The first lot was made shortly after the isolation of the organism and the second lot 2½ months later from the same strain. No difference in the biological action of these two suspensions was observed, in either normal or adrenalectomized animals. The second suspension contained 32 mg. of nitrogen per 100 cc. (estimated protein 2 gm. per liter).

This suspension was injected intraperitoneally in normal and operative control rats. Ten singly adrenalectomized rats and five other operative control rats were tested, usually 1 week after operation. Each was given intraperitoneally an injection of from 3 to 5 cc. of the streptococcus suspension. Those that received 3 cc. showed no appreciable effect. 5 cc. caused mild symptoms. After the latter dose the rats became quiet, with fur ruffled for a few hours, but at no time did they fail to react normally to blowing or other mild stimulation. For 1 or 2 days they refused to eat. All of these control animals survived (Protocol 1).

Nineteen doubly adrenalectomized rats received 3 cc. of the same streptococcus suspension. The fact should be emphasized that at the time of injection (usually 1 week after operation) the adrenal-insufficient rats used in this experiment were indistinguishable from the control animals. The former had an average weight loss of 2.8 per cent, though three of them had gained in weight since operation. All of the doubly adrenalectomized rats were killed by the injection of 3 cc. of the streptococcus suspension; the maximum time of survival was 7 hours. Some of the rats were dead an hour and a half after the injection. The average survival was 4½ hours. Protocol 2 is a typical example of this effect.

Within half an hour after injection the adrenalectomized rats were usually showing symptoms that progressed in severity until death. The animals first became quiet with fur ruffled, then failed to react to mild stimulation. Finally coma ensued and frequently the rat had one or more convulsions shortly before exitus. Respiration

became gasping and stopped before cardiac failure. Thus, the same amount of the killed streptococci produced no symptoms in control rats and invariably caused death within a maximum of 7 hours in the doubly adrenalectomized rats.

Sixteen doubly adrenalectomized rats were injected intraperitoneally with 2 cc. of the streptococcus suspension. The result in every case was fatal. One of the animals survived for 36 hours, the others all died within 18 hours. Of nine doubly adrenalectomized rats injected with 1 cc., seven died within 24 hours, one died the following day, and one recovered after a marked reaction. Three rats were adrenalectomized in two stages and all three were killed by a dose of 2 or 3 cc. of the streptococcus suspension. In fact, no rat having at least one adrenal intact has been killed by this suspension of streptococcus in amounts up to 5 cc. (the largest dose employed), and no adrenalectomized rat has survived a dose of 2 or 3 cc.

Incidental factors do not seem to be responsible for this sharp distinction in susceptibility between adrenalectomized and adrenal-intact rats.

Evidence of this fact is found in the two following examples.

Rat 139 was thyroidectomized with the removal of at least part of the parathyroids. 9 days after operation the animal had lost 17 per cent of its weight and had a persistent diarrhea. In spite of the fact that it was obviously in poor condition it was injected intraperitoneally with 2 cc. of streptococcus suspension. After this the rat became quiet, but had fully recovered by the next morning. In contrast to the preceding case, Rat 220 was in excellent condition 14 days after double adrenalectomy, being active, eating well, and having no diarrhea. It had lost 7 per cent in weight. It was injected intraperitoneally with the same amount of the streptococcus suspension (2 cc.) and was dead within 4 hours (Protocols 3 and 4).

Having established the fact that a certain quantity of killed streptococci which produced scarcely appreciable symptoms in control animals invariably killed adrenalectomized rats, we endeavored to study the mechanism of this reaction.

2. The Nature of the Toxic Substance.—The bacterial suspension was separated into two parts, (*a*) the water-clear filtrate, and (*b*) the sediment. The latter was made up to the original volume again with saline, and adrenalectomized rats were injected intraperitoneally

with 4 cc. of each of these substances. The resuspended sediment proved fatal in the same acute manner as the original suspension, the rats dying within 6 hours, while the filtrate was usually without effect. From these experiments it would seem that the toxic substance is chiefly endocellular.

The sediment fraction contained 0.2 per cent protein, total nitrogen being calculated as protein nitrogen, while the filtrate gave scarcely any color on Nesslerization. This observation suggested the hypothesis that the susceptibility to bacterial intoxication might be a non-specific protein effect. Four rats were injected intraperitoneally with 3 cc. of 2.5 to 3 per cent solutions of egg albumin, containing twelve to fifteen times the amount of protein present in the streptococcus suspension. Three of these rats survived, and one died. In the latter case a complicating uterine infection was found.

The effect of globulin was not tried. It is impossible from the data at hand to define exactly the nature of the substance in the streptococcus suspension to which adrenalectomized rats are susceptible. It seems to be chiefly endocellular and the reaction does not appear to be entirely a non-specific protein effect.

3. Chronic Intoxication.—In order to study the reaction to a more chronic form of intoxication, adrenalectomized and operative control rats were injected daily with *Staphylococcus aureus* in much smaller amounts than in the preceding experiments. The organisms used were isolated from a case of furunculosis in the outpatient department of the Peter Bent Brigham Hospital. They were suspended in saline and killed by heat. No chemical perservative was used. 1 cc. represented 1 billion staphylococci.

Eighteen rats had survived their operation for over a month. They were together in two stock cages and had been kept in this way for at least 2 weeks. All were in good condition, and it was impossible to distinguish the doubly adrenalectomized from the singly adrenalectomized animals. Each rat was injected intraperitoneally daily with staphylococcus suspension. The amount used at first was 3 cc. This was increased to 5 cc. after four injections. Under this treatment individual rats died from time to time until nine were dead.

After receiving 62 cc. of the staphylococcus suspension apiece, nine of the original eighteen rats were surviving. No rat had died for the

TABLE I.

Chronic Staphylococcus Intoxication.

Date.	Description of rat.	Weight.	No. of injections.*	Total suspension.	Result.	Adrenals.	Remarks.
1923		*gm.*		*cc.*			
Apr. 26	Tan; female.	250	1	3	Died.	Both out.	
" 30	" male.	240	5	17	"	" "	
May 1	White "	350	6	22	"	" "	Thyroid large; gastric ulcer; hydrothorax.
" 2	" "	300	6	22	"	" "	Hydrothorax; thyroid large; gastric ulcer.
" 5	" and tan; male.	220	9	37	"	" "	
" 5	" male.	175	9	37	"	" "	Echinococcus pneumonia.
" 7	" "	210	11	47	"	" "	Gastric ulcer.
" 8	Tan "	255	12	52	"	" "	
" 9	" female.	120	14	62	"	" "	
May 14	White female.	205	16	77	Survival.	Both intact.	
" 14	" and tan; female.	305	16	77	"	Left "	Early pregnancy.
" 14	" male.	305	16	77	"	" "	
" 14	" "	250	16	77	"	" "	
" 14	Tan "	195	16	77	"	" "	Echinococcus pneumonia; abscess in scrotum.
" 14	White; female.	175	16	77	"	Both "	Thyroidectomized; diarrhea.
" 14	" male.	340	16	77	"	Left "	Echinococcus cysts in mesentery.
" 14	" "	350	16	77	"	" "	" pneumonia.
" 14	" "	350	16	77	"	" "	

* Injection of *Staphylococcus aureus* (killed) suspension (1 billion per cc.). Started April 25, 1923. All surviving rats sacrificed with ether on May 14, 1923.

past 3 days and all of them seemed to be in good condition. Each of these surviving animals was given intraperitoneally 10 cc. of the bacterial suspension, which they all withstood without symptoms. 2 days after this final injection the surviving nine animals were sacrificed with ether. Every rat that died as the result of staphylococcus intoxication had had both adrenals removed. All of the animals that survived had at least one adrenal intact (Table I).

Cortex versus Medulla.

As shown in this study, the removal of both adrenals invariably leads to a remarkable decrease in the resistance of rats to bacterial intoxications. This is presumably a consequence of the loss of some function of the cortex or of the medulla, as the control operations in which this result is never seen include all the other factors of the operation, as for example traumatization of the structures in the neighborhood of one adrenal combined with the removal of the other. It seemed unlikely that the increased susceptibility of adrenalectomized rats was in any way related to epinephrine secretion. This point, however, was tested experimentally.

It appeared impossible to separate the functions of the two parts of the gland in rats by removing the medulla as Wislocki and Crowe (14) and Houssay and Lewis (15) had done in dogs. However, one adrenal was removed and the other denervated. Rats prepared in this way were injected with 3 cc. of the streptococcus suspension. They reacted like the control animals and survived this dose, which is invariably fatal to doubly adrenalectomized rats (Protocol 5). Stewart and Rogoff (16) have shown that after section of the splanchnic nerves the secretion of epinephrine is immeasurably small so it seems probable that the lack of epinephrine secretion in the adrenalectomized rats plays no part in their increased susceptibility to bacterial intoxications. The data presented do not completely rule out the possibility that the adrenal medulla, separated from its nervous connections, may react locally with the toxic substance. However, indirect evidence indicates that the increased susceptibility to bacterial or drug intoxication is related to cortical function. Thus there seems to be a parallelism in time after adrenalectomy between the recovery

of a normal resistance to morphine and the hypertrophy of accessory adrenal rests consisting solely of cortical cells (3, 7). In Lusena's experiments (8) the presence of a small amount of cortical tissue sufficed to save the adrenalectomized rats from the otherwise fatal effect of diphtheria toxin. The susceptibility of adrenalectomized rats to bacterial intoxication seems to be due to the loss of some cortical function and not to be related to a lack of epinephrine secretion.

Attempts at Protection.

Changes in the adrenal cortex caused by acute infection and especially the disappearance of its characteristic lipoids, together with its marked hypertrophy in chronic infections, seem to indicate a relative insufficiency of adrenal cortical function in pyogenic conditions. On account of its possible clinical application to human infections, then, the protection of adrenal-insufficient animals against bacterial intoxication is an important problem. The convulsions caused by the bacterial intoxication in adrenalectomized rats frequently had a prominent feature; *viz.*, a peculiar rolling character. They resemble strikingly those seen in insulin-treated rabbits. This fact suggested that the fatal effect of the bacterial injection in adrenalectomized rats might be due to hypoglycemia. However, glucose given intravenously or intraperitoneally had no protective action.

The disappearance of lipoids from the adrenal cortex naturally attracts attention to this group of substances. It is easy enough to demonstrate typical anisotropic fluid crystals in extracts of the ox adrenal. The reintroduction of the extracted lipoids in an absorbable, non-toxic form is a technical problem of great magnitude. As yet, this method, which still seems promising, has not given any conclusive results. In spite of repeated efforts, no substance has as yet been found to protect adrenalectomized rats against bacterial intoxication.

DISCUSSION.

1. The Adrenal Glands and Resistance to Bacterial Intoxication.

An interesting relationship between the adrenal cortex and the infectious process has been suspected from (1) the constancy of

adrenal lesions in acute infections, (2) the disappearance of the characteristic lipoids in acute infections, and (3) the hypertrophy of the adrenal cortex in chronic pyogenic processes. No conclusive proof, however, that the adrenal glands played any part in the resistance to intoxication by pyogenic organisms has heretofore been offered. By selecting the proper dosage of organisms, as was done in the present work, adrenal-insufficient rats can be invariably killed by a bacterial intoxication, which adrenal-intact animals always survive. Whether the cortical accessories can in time hypertrophy sufficiently to protect against bacterial intoxications as completely as the original glands has not been determined. At least 6 weeks after adrenalectomy, however, adrenalectomized rats are still susceptible to repeated injections of killed staphylococci which are harmless to control animals.

2. Protective Function of the Adrenal Cortex.

The susceptibility of adrenalectomized rats to bacterial intoxication apparently furnishes evidence of an important function of the adrenal cortex. It has long been established that this tissue seems to be essential to vertebrate life (Biedl (17)). In addition to this fact almost nothing has been known about its functions until very recently. The effect of adrenal insufficiency on heat production seems likewise to be dependent on the adrenal cortex (Marine and Baumann (18); Scott (19)). The diminished resistance of adrenalectomized rats to various drugs as reported by Lewis (3), and corroborated in the case of morphine by Scott (7), appears by analogy to be probably due also to insufficiency of the cortex alone, though this point has not been directly tested. In our experiments a denervated adrenal served to maintain the resistance of the rat to bacterial intoxication in the same manner as a gland with intact medullary secretion, while the removal of the whole adrenal profoundly affected the susceptibility of rats to bacterial intoxication. The available evidence indicates then, that resistance to bacterial intoxication is dependent upon a function of the adrenal cortex.

The susceptibility of an organism to infection is controlled by (1) the virulence of the invading organism, and (2) the resistance of the animal. With a given infection, the outcome will depend on the

resistance of the animal. The science of immunology has taught us concerning one type of resistance; *viz.*, specific antibodies. The non-specific elements in the resistance to bacterial infections and intoxications are also generally considered important, but little is known about their nature. The functional condition of the adrenal cortex seems to be an essential element in a non-specific resistance to the harmful effects of bacterial intoxications. Indeed, the reaction involved in the formation of specific antibodies may possibly impose a functional demand upon the adrenal cortex. Porak (20) states that the repeated immunization of animals in the Wassermann laboratory at Paris produced the most marked hypertrophy of the adrenal cortex that he had seen. Certainly the functional status of the adrenal cortex must be considered in future studies of resistance to infection or bacterial intoxication.

3. Detoxification or Metabolic Effect.

The early literature on adrenal function advanced the interesting but unsupported hypothesis that these glands possessed a detoxifying power in the sense that some reaction occurred between the toxic substance and an adrenal product (Oppenheim (21)). The circumstantial evidence presented to support this theory is inconclusive, and much of it has been discredited (Tawara (22); Exner (23)). That the protective function of the adrenal glands is due to a detoxication, in accordance with this hypothesis, seems unlikely. The alternative explanation that the susceptibility of adrenalectomized animals is due to a fundamental metabolic effect of the adrenal insufficiency appears to correspond more closely to the type of reaction observed. By using very small doses of bacteria, the same profound, prolonged disturbance in the condition of the adrenalectomized rat can be produced that was noted previously as an effect of morphine. Such animals ate little and showed a greatly diminished activity, finally dying after a number of days. The susceptibility of an adrenal-insufficient animal to bacterial intoxication seems to be dependent on some function of the adrenal cortex. Whether the state is consequent on a lack of detoxification by the adrenal cortex, or comes about indirectly through the effect of adrenal insufficiency on some other tissue is being investigated.

4. Extreme Susceptibility of a Small Group.

A few doubly adrenalectomized rats chiefly belonging in the group of 10 per cent that have a severe reaction to adrenalectomy, yet survive 2 weeks or more, exhibit a greatly reduced resistance to substances that are not toxic to most adrenalectomized animals. Examples of this extreme sensitivity are afforded by the rat killed by the intraperitoneal injection of egg albumin, and the one in which the clear filtrate from the streptococcus suspension produced an intoxication fatal after 4 days. Because of such instances the minimum lethal dose is an unsatisfactory standard whereby to judge the susceptibility of adrenalectomized rats. The diminished resistance to bacterial intoxication is not an unusual reaction of the sort mentioned, for every adrenalectomized rat shows it.

5. Exhaustion of Adrenal Accessory Lipoids.

No systematic study of the histological appearance of the accessory cortical rests was made. However, a marked difference was observed between the cortical accessory cells of adrenalectomized rats (1) that were sacrificed, and (2) that were killed by a chronic bacterial intoxication. In the former the cells showed the typical honeycombed appearance (hematoxylin and eosin preparations) signifying abundant lipoid granules, while in the latter the cells were shrunken, pycnotic, and not honeycombed. This is the same type of change seen in the adrenal cortex in pyogenic infections, and it furnishes further evidence in correlation of the results of bacterial intoxication with the disappearance of lipoids from the adrenal cortical cells. This again raises the important question whether the lipoid granules are the precursors of an internal secretory product of the adrenal cortex, or possibly the menstruum of the active principle. No direct proof of such a secretion has been brought. Yet the evidence of Dietrich (24) is highly suggestive of it. He observed diminution in size and peripheral (juxtavascular) arrangement in the cell of the lipoid granules as the first stage of lipoid disappearance accompanying infected war wounds. Fatal wounds unaccompanied by infection were invariably associated with a large amount and uniform distribution of lipoid granules in the cell.

CONCLUSION.

1. The resistance of rats to bacterial intoxication is greatly decreased after double adrenalectomy.

2. This decreased resistance is dependent upon a functional insufficiency of the adrenal cortex.

3. A dose of killed streptococci or staphylococci can be obtained that is invariably fatal to adrenalectomized rats, before hypertrophy of cortical accessories, but never kills control rats.

I wish to acknowledge my indebtedness to Dr. David Marine who first suggested to me the importance of the adrenal cortex in the defensive mechanism against harmful biological processes, and also to express my appreciation to Dr. A. W. Sellards and Dr. W. B. Cannon for advice in this investigation.

Control.

Protocol 1.—Rat 70. White, female.

Nov. 24, 1922. Individual cage. Active.

Nov. 27. Active, eating well, weight 197 gm. Operation: excision of right adrenal. Easy operation. Moderate fat.

Dec. 4. Active, eating well, weight 195 gm. At 11 a.m. injected 5 cc. of streptococcus suspension (35 billion per cc.) intraperitoneally. Became somewhat quiet, but reacted vigorously to blowing. 5 p.m. Fairly active, fur slightly ruffled.

Dec. 5. Active, but not eating.

Dec. 7. Active, beginning to eat. Weight 179 gm.

Jan. 8, 1923. Active, weight 217 gm.

Adrenal Insufficiency.

Protocol 2.—Rat 87. Tan and white, male.

Dec. 6, 1922. Individual cage.

Dec. 11. Active, eating well. Operation: excision of both adrenals.

Dec. 18. Active, eating well, weight 340 gm. At 10.30 a.m. injected 3 cc. of streptococcus suspension (35 billion per cc.) intraperitoneally. 1.30 p.m. Semicomatose. Dead at 2 p.m.

Autopsy.—Thyroid large and red. Heart and lungs normal. Abdomen: about 2 cc. of free fluid. Adrenals absent. No accessories seen. Much retroperitoneal fat. Stomach: mucous membrane smooth.

Control (Thyroidectomy).

Protocol 3.—Rat 139. White, female.

Feb. 10, 1923. Active, weight 187 gm. Operation: thyroidectomy and partial parathyroidectomy. Severe hemorrhage.

Feb. 12. Rather quiet. Diarrhea. Not eating well.

Feb. 19. Diarrhea has been persistent since operation. Quiet. Evident loss of weight. Muscle tone poor. Weight 155 gm. At 11.30 a.m. injected 2 cc. of streptococcus suspension (31 billion per cc.) intraperitoneally. At 5.30 p.m. quiet. Reacts briskly to blowing. Diarrhea. 9 p.m. alert.

Feb. 20. Quiet but alert. Diarrhea. Ate nothing yesterday.

Feb. 21. Fairly active. Eating well. Diarrhea unchanged.

Apr. 5. In good condition.

Adrenal Insufficiency.

Protocol 4.—Rat 220. White, female.

Apr. 17, 1923. Active, weight 165 gm. Operation: double adrenalectomy. Easy operation.

May 1. Active, eating well, weight 153 gm. At 1.06 p.m. injected 2 cc. of streptococcus suspension (31 billion per cc.). 2.30 p.m. Quiet. Found dead at 5 p.m.

Autopsy.—Thyroid: moderate size. Heart and lungs normal. Abdomen: adrenals out. Right kidney adherent to the liver. Much retroperitoneal fat. Stomach: congestion of mucous membrane. No ulcers.

Denervation of Adrenal.

Protocol 5.—Rat 237. White, female.

Apr. 28, 1923. Active, weight 218 gm. Operation: excision of right adrenal; denervation of left adrenal.

May 3. Active, weight 210 gm. At 12 noon injected 3 cc. of streptococcus suspension (31 billion per cc.) intraperitoneally. No symptoms noted during the afternoon.

June 1. Active, eating well.

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THE EFFECT OF PILOCARPINE ON THE NUMBER OF SMALL LYMPHOCYTES IN THE CIRCULATING BLOOD FOLLOWING LIGATION OF THE THORACIC DUCT.

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INTRODUCTION.

In a previous communication the writer has shown that following the intrathoracic ligation of the thoracic duct in cats, the number of small lymphocytes in the circulating blood is temporarily decreased (Lee (1922, *a*)). In view of this observation experiments were undertaken to determine what effects an artificially produced lymphocytosis would have upon the number of small lymphocytes in the blood after the thoracic duct had been tied.

Materials and Method.

In this series of experiments eight adult male cats were used, of which number two served as controls. The absolute number of small lymphocytes in the blood was calculated from the total white blood cell determination and from a differential count of 300 or more cells from a smear stained with Wright's blood stain. Specimens of blood were examined before the ligation of the thoracic duct, and at definite intervals following the intraperitoneal administration of pilocarpine nitrate as shown in Table I. The thoracic duct was ligated in the chest under sterile precautions according to the method previously described (Lee (1922, *b*)).[1] About 24 hours later pilocarpine nitrate, sterilized by boiling gently for 5 minutes in a freshly prepared aqueous solution, was injected into the peritoneal cavity to the extent of 3.3 mg. per kilo of body weight. Food was withheld for a period extending from 24 hours before the ligation of the thoracic duct until 24 hours after the administration of the pilocarpine; water, however, was always provided. From 2 to 3 days after the injection of pilocarpine the animals were placed under ether anesthesia, and the mesenteric lymph vessels were injected in a centripetal

[1] All operations were performed under ether anesthesia.

direction in order to determine whether the thoracic duct had been completely ligated. Tissue from the animals was placed in 10 per cent commercial acid formalin and subsequently sectioned in paraffin. The postoperative course of all the animals showed that they were free of infection.

OBSERVATIONS.

Aside from minor variations the results were comparatively uniform. The following protocol and Table I, together with Text-fig. 1, represent an average case.

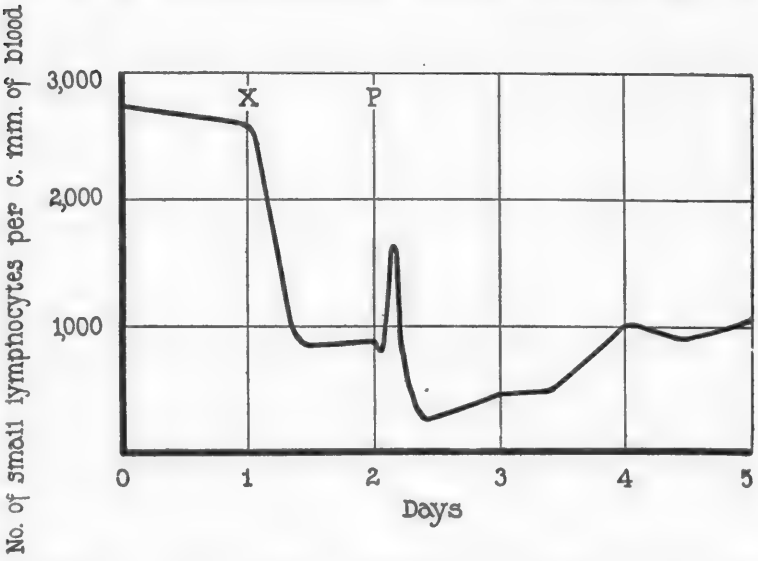
TABLE I.

Date.	Time.	Total white blood count per c.mm.	Polymorphonuclear neutrophils.	Eosinophils.	Small lymphocytes.	Large lymphocytes.	Large mononuclears.	Transitionals.	Absolute No. of small lymphocytes per c.mm.
1923			*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	
Apr. 23	10.00 a.m.	13,920	68.7	7.7	19.7	1.7	1.0	1.3	2,742
" 24	10.00 "	12,800	61.7	11.7	20.7	3.0	0.7	2.3	2,649
	11.00 "	Ligation of thoracic duct.							
	5.00 p.m.	16,900	90.7	0.3	5.3	1.0	1.0	1.7	896
" 25	10.20 a.m.	16,000	88.0	0.3	6.0	1.7	1.7	2.3	960
	10.30 "	8.8 mg. of pilocarpine nitrate injected intraperitoneally.							
	11.30 "	8,940	85.7	0.3	9.0	1.3	1.7	2.0	803
	12.45 p.m.	23,500	90.0	0	7.0	0.3	1.0	1.7	1,645
	2.00 "	12,000	90.7	0.3	5.7	1.0	1.3	1.0	684
	3.15 "	10,200	91.7	0	4.0	0.3	1.7	2.3	408
	4.45 "	10,080	93.7	0.3	2.3	0.3	1.3	2.0	231
" 26	10.00 a.m.	8,800	86.7	1.0	5.7	1.7	1.0	4.0	501
	4.30 p.m.	6,020	77.7	9.3	8.7	2.0	1.0	1.3	513
" 27	9.30 a.m.	7,600	71.3	9.3	14.7	1.0	1.7	2.0	1,017
	5.00 p.m.	7,100	72.7	8.7	13.0	1.7	3.0	1.0	923
" 28	9.00 a.m.	6,200	67.3	9.0	19.0	1.3	1.0	2.3	1,178

Cat 1.—Adult, gray, male cat; seemingly in good health; weight 2,580 gm. Aseptic intrathoracic ligation of thoracic duct on April 24. Intraperitoneal injection of 8.8 mg. of pilocarpine nitrate on the following day. This injection was followed by vomiting, diarrhea, sweating, lacrimation, and pronounced salivation; respiratory distress was not observed. It was difficult to secure blood for the two counts immediately following the injection. On the 3 following days the animal was in good condition; it ate some meat on the 2nd day after the administration of pilocarpine. On April 28 the mesenteric lymph vessels were injected in a centripetal direction with Berlin blue while the cat was under the influence of ether; lymph vessels to the liver and kidneys were markedly en-

larged; some retrograde injection of the mesenteric lymph vessels occurred; a slight amount of free fluid was found in the peritoneal cavity; the thoracic duct was definitely ligated; pieces of the various organs were placed in formalin for histological examination.

In this cat the increase in the absolute number of small lymphocytes in the circulating blood after the administration of pilocarpine was 104 per cent; the average increase for the six animals was 98.7 per



TEXT-FIG. 1. Curve to show the changes in the absolute number of small lymphocytes in the circulating blood of the cat, following the intrathoracic ligation of the thoracic duct (at *X*), and the intraperitoneal administration of pilocarpine nitrate (at *P*).

cent, whereas the increase for the two control cats was 100.8 per cent. The peak of increase was found to come within 2 hours after the injection, and in two animals a secondary rise appeared after the initial drop. However, the low point of the decrease following the peak was, as a rule, lower than the point from which the initial rise began; and from this low point the curve invariably went upwards and simulated the rise seen after the ligation of the thoracic duct.

Frequently the mesenteric lymph vessels were collapsed and difficult to inject at first; but after the injection was once successfully begun the other mesenteric lymph vessels became dilated because the injection mass displaced the lymph in the lymph glands and larger channels and thus forced it in a peripheral direction. In this dilated condition the lymph vessels were frequently injected in a retrograde manner.

The abdominal lymph glands, particularly the mesenteric glands, were always large and edematous. The lymph trunks leading to the liver and iliac glands were also greatly distended. On section the lymph glands yielded a large amount of clear, watery fluid with numerous small, fat-like droplets floating on the surface. Although the peritoneal cavity did not contain any excess of fluid, nevertheless the loose retroperitoneal tissue was edematous. No edema of the extremities or of the subcutaneous tissues was observed.

Examination of the histological material showed definite changes, primarily in the abdominal lymph glands and in the kidney. The most striking feature of the lymph gland was the enormous dilatation of the lymph channels, particularly in the medullary portion of the gland. The distention made the so called reticulo-endothelium appear as a stretched network of cells whose processes communicated with one another, forming meshes within which the injection mass was found. Possibly in a vitally stained animal the distention of the lymph sinuses may be a method for throwing some additional light on the interesting questions relating to the nature of the cells lining these sinuses.

Of unusual interest was the appearance of the kidney. Under low magnification numerous clear spaces of various sizes were seen throughout the kidney substance, but localized chiefly in the cortex. Higher magnification showed that these clear spaces were cross-sections of thin vessels of which the endothelium was stretched and the nuclei narrow and flattened. These vessels were numerous near glomeruli and along the medullary rays, and were undoubtedly lymph vessels. Examination of the individual cells of the tubules showed that a high degree of vacuolation existed in all the cells with the exception of those of the collecting tubules. The vacuoles were small, but numerous, and although found throughout the cell they were more numerous near the nucleus. They represented fat dissolved out during the

preparation of the section. The glomeruli showed no vacuolation and Bowman's capsule was not abnormally distended; no excess of fluid was collected in the tissue spaces.

DISCUSSION.

Although the original observation of Horbaczewski in 1891 that pilocarpine produces a marked leucocytosis has been repeatedly confirmed by subsequent investigators (Harvey; Lefmann; Waldstein; Schenk; Skórczewski and Wasserberg; Bertelli, Falta, and Schweeger) nevertheless other observers (Wollenberg; Platz) who were interested primarily in the effects of the nervous system on the blood picture were unable to obtain an outspoken lymphocytosis. Certain it is that studies of the action of pilocarpine made with the aid of thoracic duct fistulæ have conclusively shown that this drug causes a marked increase in the number of lymphocytes in the thoracic duct lymph and in the blood (Rous; Dixon (1909)).

Inasmuch as all the animals in this series showed a definite lymphocytosis, it is believed that the massive dose administered was instrumental in producing the result. The comparatively small dose of 0.01 gm. given intramuscularly or even subcutaneously to patients frequently produced equivocal results, even though such general symptoms of pilocarpine activity as salivation, lacrimation, and sweating were manifest (Wollenberg). The largest doses administered were those of Ruižcka, who gave 50 mg. of pilocarpine per kilo of body weight subcutaneously to dogs and obtained a rise in the leucocytes equal to thirty-two times the original amount. The smallest doses were given by Port and Brunow, who found that even 0.0007 gm. per kilo of body weight caused a rise in the absolute number of lymphocytes in the blood of dogs.

The intraperitoneal method of injection was chosen because of its simplicity and convenience. That the absorption of the drug from the peritoneal cavity was comparatively slow was shown by the fact that the full effect on the blood picture was not observed in some cases until the end of 2 hours. It is important to correlate the method of administration with the time of blood examination, and it is needless to point out that a single count after the drug has been given (Harvey) is not a fair criterion of its action.

Even with large doses the action of the drug was selective; vomiting occurred only early, lymphocytosis was manifest late, while salivation came on quickly and was the last symptom to disappear. This sequence of symptoms suggests either that some tissues respond more quickly than others to the direct action of the drug, or that certain organs, like the adrenal, are affected and that these in turn liberate a secretion which produced pharmacological changes.

The lymphocytosis produced may have been caused in several ways. In the first instance the pilocarpine may have caused an absolute increase in the number of lymphocytes at their centers of production. But against this view must be brought the observation that the lymphocytic response to the intravenous administration of the drug took place within 10 minutes, and it is difficult to believe that a large number of lymphocytes could have been liberated in such a short space of time, all the more so because histological examination of the spleen and lymph glands failed to show a definite increase in the number of mitotic figures.

In the second place, the lymphocytosis may have been produced by the direct passage of lymphocytes into the blood stream at their centers of formation. Since this communication was not prevented by the ligation of the thoracic duct, a relatively great lymphocytosis might have been expected after the injection of pilocarpine. Although this marked lymphocytosis never occurred, the method of passage is not entirely excluded (von Schumacher).

Finally, pilocarpine through the contraction of the plain muscle it causes in various organs may have mechanically produced a lymphocytosis by a discharge of lymphocytes first into lymph vessels and secondarily into the blood stream. Harvey originally advanced this explanation, and his view has subsequently received support from the work of Rous and of Dixon (1912). The experiments here recounted also contribute to Harvey's view. For it was found that after the intraperitoneal injection of pilocarpine the same *relative* increase in the absolute number of small lymphocytes in the blood stream occurred whether the thoracic duct was ligated or not. To this may be added the observation that pilocarpine caused not only a rise in the total number of lymphocytes, but that it also caused a drop to follow the rise, and this drop not only invariably reached the level

from which the rise began, but in half the experimental animals as well as in the two controls it went even lower with the result of a typical negative phase after the positive rise. This suggests that the drug had caused a mechanical discharge of the lymphocytes from their seats of formation with result later in a temporary lack of them. It would seem that the ligation of the thoracic duct shut off the normal avenue into the blood for about 58 per cent (the mean for the six animals) of the small lymphocytes, leaving 42 per cent to be supplied by that portion of the lymph system not obstructed by the thoracic duct ligation; and it was this latter portion of said system which responded in the same relative degree to pilocarpine as did the entire intact lymph system in the control animals. The observations do not accord with Harvey's view that the spleen is the main source for the lymphocytic increase, but indicate to the contrary that the organ is no more specialized for the production of lymphocytes than is any other part of the lymphopoietic system.

As regards the other blood cells which have come under investigation it may be said that there occur changes in the number of polymorphonuclear neutrophils after pilocarpine paralleling the changes in the number of small lymphocytes. The eosinophils were practically absent following the ligation of the thoracic duct, and the administration of pilocarpine did not cause them to appear. The other cell groups remained essentially unchanged.

SUMMARY.

In a series of animals in which the thoracic duct had been tied it was found that the relative increase in the number of small lymphocytes in the circulating blood following the intraperitoneal administration of pilocarpine nitrate was the same as for the control animals. While support is brought for Harvey's view that pilocarpine causes a lymphocytosis through the contraction of plain muscle, on the other hand evidence is presented which indicates that the spleen is no more specialized in the production of small lymphocytes than any other portion of the lymphopoietic system.

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STUDIES ON THE RELATION BETWEEN TUMOR SUSCEPTIBILITY AND HEREDITY. I.

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INTRODUCTION.

The failure of the many attempts to demonstrate a parasitic origin for neoplastic disease in mammals has shifted attention to other possible factors in its etiology. One of these is the relation between tumor growth and heredity. Although mammals offer rather poor material for research in genetics on account of the small number of offspring produced, the demonstration of the histological and biological similarity of the neoplasms of man and rodents has led to the selection of the latter for experimentation.

Studying the inoculated tumor in mice, Tyzzer and Little[1] and Little[2] investigated the inheritance of conditions permitting the growth of an implant and have obtained results which are compatible with an explanation on the basis of multiple factors. The study of the spontaneous tumor as a close parallel to the condition found in man, has attracted a series of investigators. An early communication by Thorel[3] simply reported the occurrence of a high tumor rate observed in a stock

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of laboratory mice. Tyzzer[4] from his observations upon mice with cancerous ancestry (mainly primary lung tumor) concluded that heredity plays a part in cancer incidence. Murray[5] from a biometrical treatment of groups of mice with mammary gland tumor in which he compared females from mothers or grandmothers which had cancer to those with more remote cancerous ancestry, reached the same conclusion. Slye[6] and Loeb and Lathrop[7] have raised strains of mice showing an extraordinarily high cancer incidence, and Loeb has maintained groups with high and low rates at fairly constant percentages for a number of generations. Slye states that she has raised four strains from three to five generations without cancer. The ages of the individuals are not given. These facts and certain crosses that have been made, have been interpreted as meaning that hereditary units traveling in the germ plasm of the different strains, persistently express themselves in the constitutions of the individuals of each succeeding generation.

Little and his collaborators[8] in a resumé of data upon human beings, collected by the Eugenics Record Office at Cold Spring Harbor, makes a comparison of the cancer incidence among persons having a cancerous parent, to the incidence in the population at large, and finds that the disease is much more frequent among individuals of cancerous parentage than would be expected according to the law of probability. In statistics upon cancer incidence among human beings some allowance must be made for uncertainties in diagnosis.

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[8] Inheritance of a tendency to "Cancer" in man, *Carnegie Institution of Washington, Year Book No. 20*, 1921, 139.

It was with the aim of securing Mendelian data that we planned a series of crosses involving the hybridization of different strains of mice. Unfortunately several epidemics of so called mouse typhoid ravaged the major portion of the older groups. From the fragment that remained, the data, while as yet incomplete, have furnished some facts sufficiently interesting to warrant a preliminary presentation of the subject at this time.

Much of the confusion which has characterized the discussion of the inheritability of a tendency to cancer, has arisen from a certain vagueness connected with the connotation of "heredity." In recent years, the confirmation and elaboration of the Mendelian law has not only clarified our conception of heredity, but furnishes definite tests which may be applied practically, at least in the case of the lower animals. The essential characteristic of Mendelism, *i.e.* segregation of the hereditary units in the germ cells, can be clearly seen in the offspring from hybrids of known ancestry. Therefore, the breeding test to be applied consists of making up hybrids and then analyzing what has occurred by inbreeding or backcrossing. To investigate any particular character, an individual possessing it is crossed with one that does not have it, and whose ancestors were free from it. The offspring from such a cross are usually all alike in either having or not having the character. If it appears in the first filial generation it is termed dominant, if not, it is called recessive. The following diagram may serve to illustrate these facts.

P_1 $\quad \frac{D}{D} \times \frac{R}{R}$

Germ cells D, D R, R.

F_1 $\quad \frac{D}{R} \quad \frac{D}{R}$

Germ cells D, R D, R.

F_2 $\quad \frac{D}{D} \quad \frac{D}{R} \quad \frac{R}{D} \quad \frac{R}{R}$

Since the germ cell is duplex in nature, the dominant and recessive parents are represented by the formulæ $\frac{D}{D}$ and $\frac{R}{R}$, respectively. These individuals can produce egg or sperm of but one kind (either D or R). Fertilization of one by the other results in individuals $\frac{D}{R}$ which by definition look like the parent with the dominant character, although they carry the recessive gene also. This generation produces germ cells of two kinds, D and R, so that random fertilization gives four combinations, three of which are individuals showing the dominant character, and one is

recessive. Of the dominant, however, two carry the recessive latent gene and are termed heterozygous.

If the F_1 generation, instead of being inbred, is backcrossed to either type of parent, a different ratio is obtained in the F_2.

F_1	$\frac{D}{R}$	×	$\frac{D}{D}$ (Parent).
Germ cells	D, R		D, D
Backcross	$\frac{D}{D}$ $\frac{D}{R}$		$\frac{D}{R}$ $\frac{D}{D}$

As illustrated above, if the backcross is made to the parent with the dominant character, the resulting offspring are all of the dominant type, a higher percentage than in the second filial generation from the first cross and equal to that of the first filial generation. If the backcross is made to the recessive parent, half of the progeny will be dominant (heterozygous) and half recessive—giving a smaller percentage of dominants than when the F_1 is inbred.

These ratios are obtained when a character is dependent on one gene in the germ cell. If two genes are concerned the parents may be represented by the formulæ $\frac{DA}{DA}$ (dominant) and $\frac{RB}{RB}$ (recessive). Their offspring will be heterozygous $\left(\frac{DA}{RB}\right)$ dominants capable of producing four types of germ cells, DA, DB, RA, RB. If there is no linkage the four kinds will be produced in equal numbers.

P	$\frac{D}{D}$ $\frac{A}{A}$		×	$\frac{R}{R}$ $\frac{B}{B}$	
F_1	$\frac{D}{R}$ $\frac{A}{B}$				
Germ cells	**DA**	**DB**	**RA**	**RB**	
F_2	$\frac{D}{D}$ $\frac{A}{A}$	$\frac{D}{D}$ $\frac{B}{A}$	$\frac{R}{D}$ $\frac{A}{A}$	$\frac{R}{D}$ $\frac{B}{A}$	
	$\frac{D}{D}$ $\frac{A}{B}$	$\frac{D}{D}$ $\frac{B}{B}$	$\frac{R}{D}$ $\frac{A}{B}$	$\frac{R}{D}$ $\frac{B}{B}$	
	$\frac{D}{R}$ $\frac{A}{A}$	$\frac{D}{R}$ $\frac{B}{A}$	$\frac{R}{R}$ $\frac{A}{A}$	$\frac{R}{R}$ $\frac{B}{A}$	
	$\frac{D}{R}$ $\frac{A}{B}$	$\frac{D}{R}$ $\frac{B}{B}$	$\frac{R}{R}$ $\frac{A}{B}$	$\frac{R}{R}$ $\frac{B}{B}$	

With random fertilization there will be sixteen classes of offspring of the second filial generation. Every class which contains at least one "dose" of both D and A

will produce the dominant type. There are nine dominants to seven recessives. But the backcross to the homozygous dominant parent will give a larger and to the recessive parent a smaller proportion of animals showing the dominant character.

F_1 $\frac{DA}{RB} \times \frac{DA}{DA}$ (Parent).

Backcross $\frac{DA}{RB}$ (dominant), $\frac{DA}{DA}$ (dominant), $\frac{DA}{DB}$ (dominant), $\frac{DA}{RA}$ (dominant).

Likewise when the character depends on three or four more genes, different ratios will be obtained in the F_2, but the backcross to the dominant and recessive types will yield larger and smaller numbers of dominants, respectively, than obtained from the inbred F_1. These ratios, therefore, constitute a check upon the data, and may be used as controls in an experiment.

It is possible that tumor susceptibility is not inherited according to the simple schemes outlined above. Morgan has suggested that it might be due to a somatic mutation. In that case, while still Mendelian, more complicated relationships would be indicated. It is not necessary to discuss these further possibilities until the simpler explanations have been tested.

The Tumor.

The work more especially of Slye, has indicated that tumors of particular types or particular organs are inherited as separate units. Slye found that a number of neoplasms of one type can be concentrated in one (or a few) strains of mice, while other strains may be free from them. In the present paper one type only will be considered—that which is reported to be the most common among mice, namely the mammary gland tumor. It usually presents itself as an adenocarcinoma, or carcinoma. No attempt has been made to subdivide further these growths, and classify them as different units. Autopsies are made upon all mice that die, and all tumors are verified from the microscopic examination of sections.

When the character dealt with is a neoplasm, certain complications are introduced into the situation. (1) In the stocks under observation adenocarcinoma has appeared only in the female sex. Slye has reported sarcoma and carcinoma of the mammary gland as occurring in the male, but it is not common, and in the strains used in these

experiments neither sarcoma nor carcinoma has been found in the mammæ of the male mouse. (2) Variability. Susceptibility to neoplastic disease, in mice as in human beings, is characteristic of middle or old age. While a cancer *may* appear in a mouse as young as $4\frac{1}{2}$ months, the average tumor age in many races is $1\frac{1}{2}$ years, and sometimes the disease does not make its appearance until the individual is 2 or 3 years old. At 2 years a female mouse has passed the reproductive period and is considered old. Many mice may live to the comparatively advanced age of 2 years and die without producing a tumor, which, had they lived a few months longer might have been classed as tumor mice. The fact that a mouse dies without showing a tumor does not necessarily mean that it was incapable of producing one. Tumor incidence in any strain is probably always higher than statistics would indicate. From these facts it is readily seen that tumor susceptibility must be regarded genetically as a variable character. (3) Breeding. Mice with tumors are difficult to breed, not only on account of their age, but often their poor health seems to prevent reproduction. In practice the young adults for breeding are selected from strains of high tumor incidence on the chance that the individuals so chosen will eventually develop tumors.

EXPERIMENTAL.

At the time these experiments were commenced, we were in possession neither of a strain very rich in tumor mice, nor one which was known to be tumor free, but at the same time that we were endeavoring to build up such races, a tentative beginning was made on the problem by outcrossing strains from different sources.

In all cases, the female parents were taken from the tumor stocks which had been acquired by purchase from the Lathrop Mouse Farm. These strains had been under observation for some time, and though none showed a very high tumor incidence, individuals were chosen from those giving the highest rate and also, as far as possible, from mothers which had developed tumors. For the male parents various stains were used. One was obtained through the courtesy of Dr. C. C. Little from the Carnegie Institute at Cold Spring Harbor. While it was not guaranteed as a non-tumor stock, it had been in Dr. Little's possession for 7 years; fairly large numbers had been raised, some of the females attaining old age, and no external tumors had ever been found. Males were used also from the pink eyed, brown spotted race which had been under observation by Dr. Detlefsen at

the University of Illinois, for 4 years, without any tumors having been observed. The strain of albinos procured from Dr. Bagg at the Cancer Memorial Hospital is known to have produced a few mice with mammary gland cancer, but the incidence is probably low. (It is still under observation.) Five agouti males caught at Bronx Park were used on the supposition that they were wild. One of them, however, when mated with albinos, gives albinos in the F_1, suggesting that its ancestry may include an escape from the laboratory and that it is not wild. The two caught at Staten Island and in New York City are probably really wild stock.

TABLE I.

Fifteen crosses between females from high tumor strains and males from outside sources. In the third column opposite each male is listed the number of his daughters which had not developed tumors at 10 months of age (or later) and in the fourth column the number of his daughters which did have tumors.

Catalogue No. of male.	Source of male parent.	No. of daughters without tumor.	No. of daughters with tumor.
15-47	Little.	8	4
16-45	"	8	3
16-46	"	4	3
16-04	Detlefsen.	10	5
15-69	"	3	0
15-71	"	0	1
15-97	"	1	0
17-82	Bagg.	2*	1
17-76	"	5*	1
17-92	"	0*	2
18-97	"	2*	7
18-37	Bronx.	0*	1
18-64	"	2*	1
18-63	Staten Island.	0*	1
19-83	New York.	0*	1

* Many daughters still living.

The results of the crosses are given in Table I. Many losses occurred in all generations, not only from the usual causes incident to mouse culture, but also from the series of typhoid epidemics. In certain cases, whole groups were wiped out, including parents and offspring. If the latter had not reached tumor age, the entire group is not reported. Also certain experiments begun somewhat later than the rest, in which the F_1 are just reaching tumor age, are not given here. With these exceptions, Table I includes *all* outcrosses which have been

made, giving all females in the F_1 which reached the age of 10 months or more.

It is to be noted that one or more of the daughters developed tumors in every cross here listed, with two exceptions. In one of these cases Male 15-97 had but one daughter which lived to be as old as 10 months, in the other, Male 15-69, there were but three females. If a tendency to cancer is hereditary the immediate appearance of tumors in the F_1 indicates either that tumor susceptibility is dominant or that none of the males used as parents were non-tumor mice. If tumor susceptibility is dominant, a female which develops a tumor may have the formula of either $\frac{D}{D}$ (homozygous) or $\frac{D}{R}$ (heterozygous), but it is impossible to tell by inspection to which type she belongs. If she

TABLE II.

The distribution of tumors among the daughters of tumor mice obtained from the stock room.

Catalogue No. of the mouse.	Non-tumor daughters.	Tumor daughters.
H5	5	2
H8	3	1
H11	2	0
H13	1	0
H15	0	1

were $\frac{D}{D}$ all her daughters should inherit tumor susceptibility, if $\frac{D}{R}$, half of them would receive it, but since the character is, in addition, variable, even that 50 per cent might not actually develop neoplasms. Therefore, the two exceptions cited above, in which one and three daughters did not show tumors, are not adequate tests for dominance and may be disregarded. As for the other case, the chances are enormously against finding 100 per cent tumor mice in a random selection of thirteen males from six different sources.

It will be interesting to compare these data with another group of individuals which have come under observation. From time to time, females with tumors have been brought to us from the mouse breeding station of The Rockefeller Institute. Five of these

were pregnant when brought in. In the stock from which they came the old females are continually discarded, so that the average age is below tumor age, and the tumor rate is not known. It is possible that they had mated with tumor mice. Nevertheless it is of interest that three of them had tumor mice among their daughters and the other two had too small a number of offspring (two and one females, respectively), to be regarded as contradictory evidence. An examination of the daughters of tumor mice in our own inbred supply stock, does not reveal any conflicting data. Usually only a small number of daughters lived to tumor age. Heterozygosity of the mother and the variability of the character would necessitate a fairly large number of daughters to form a decisive test. In the one instance in which eleven females lived more than 12 months, only one had a tumor, which suggests that the degree of variability is great or that more than one gene is involved.

From the first seven crosses given in Table I, the majority (though not all) of the descendents have reached tumor age. Unfortunately, both of the stocks (Little and Detlefsen) from which the male parents were taken were lost before giving sufficient data to prove that they were non-tumor stocks (or individuals), but the F_1 as well as having been inbred were backcrossed with the tumor stock. If tumor susceptibility is hereditary and dominant, a higher percentage of tumor mice should appear from the backcrossed than from the inbred parents, and a comparison of the two rates should function as a control for the experiment. A summary of the data from these two generations, as well as from the F_1, and the female parents used for both the original and backcrossed mothers is given in Table III.

The females selected for the original cross showed a slightly smaller percentage of tumors (7 out of 26) than did the backcross mothers, where about one-third (19 out of 56) proved to be tumor mice. As before stated, however, many of those which did not succumb to neoplasms may genetically have been tumor mice—even homozygous, without developing a tumor. In the F_2, 12 mice out of 89 (a 1:6.5 ratio) had tumors, and the backcross daughters gave 50 tumors in a total of 159 mice (about 1:2). thus fulfilling the expectation. If we note only those offspring of which the mothers (original mothers and backcross mothers) were tumor mice we find for the F_2, 6 tumors in

30 mice (1:4) and for the backcross 13 tumors in 39 mice a (1:2 ratio). Here again the backcross gives more tumors than the inbred generation. The backcross ratio equalled the F_1, which showed 16 tumor mice in 50 (1:2). Since the male parents have not been proven non-tumor individuals the F_2 ratio of 1:6.5 cannot be used as a basis for calcu-

TABLE III.

Results of experiments in each of which one male was crossed with several females from tumor stock and his sons backcrossed to females from tumor stock. The lists show how many daughters (F_1), granddaughters (F_2), and daughters from the backcross did or did not have tumors.

Catalogue No. of male parent.	No. of female parents (P) without and with tumor.		No. of daughters (F_1) without and with tumor.		No. of granddaughters (F_2) without and with tumor.		Backcross mothers.		No. of daughters from backcross without and with tumor.	
	Without.	With.	Without.	With.	Without.	With.	Without.	With.	Without.	With.
15-47	3	4	8	4	53	5	23	11	69	24
16-04	7	0	10	5	13	3	7	1	13	6
16-46	1	2	4	3	5	4	7	6	25	20
15-69	1	1	3	0	0	0	0	1	2	0
15-71	1	0	0	1	0	0	0	0	0	0
15-97	1	0	1	0	1	0	0	0	0	0
16-45	5	0	8	3	5	0	0	0	0	0
	19	7	34	16	77	12	37	19	109	50

TABLE IV.

The distribution of tumor mice among F_2 and backcross females classified according to whether or not they have been bred.

	Not bred.		Bred.	
	Non-tumor.	Tumor.	Non-tumor.	Tumor.
F_2	9	1	19	2
Backcross	15	5	20	10

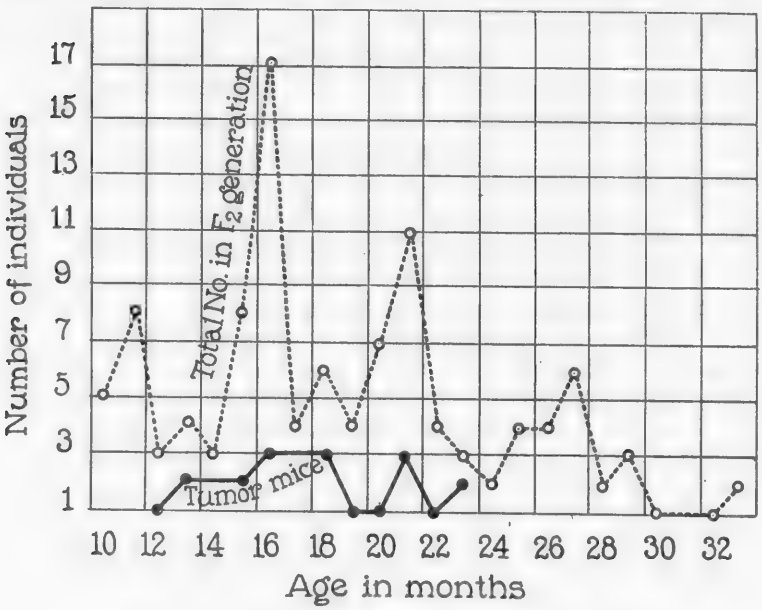
lating the number of genes responsible for the character. The comparison of the F_2 and backcross is, however, valid evidence for the inheritability of cancer susceptibility, but the figures should probably be modified by a consideration of the environmental factor of irritation.

The word "susceptibility" as applied to the inheritance of tumor growths, doubtless connotes in the minds of many investigators the tendency on the part of animal tissue to respond by producing abnormal growths when subjected to various stimuli, known or unknown (chemical, mechanical, etc.). This was suggested by the prevalence of certain types of neoplasms among classes of people such as paraffin workers, chimney sweeps, betel-nut chewers who are accustomed to localized chronic irritation, and the experimental work done upon the artificial production of tumors by means of parasites, coal tar, etc., has emphasized the importance of this aspect of the situation. In the case of cancer of the mammæ in mice, breeding and rearing young has been regarded as the contributory factor. It is not an absolutely necessary one since mice which have never had young do produce tumors, but Lathrop and Loeb[9] have published percentages indicating that in non-breeding mice the tumor rate was, on the whole, somewhat lower and the tumor age higher than in breeding mice. The amount of decrease varied in different strains. In only one strain was the tumor rate higher in the non-breeding than in breeding mice; in the other eight strains tested it was lowered, sometimes slightly, sometimes to a considerable extent. There is a little evidence in our stocks which seems corroborative, but the point is being further investigated. In the data here presented there are two small classes of bred and non-bred mice. Among the non-bred, in the F_2 there was 1 tumor mouse to 9 without tumors, as against 5 tumor mice to 15 without tumor in the backcross.

In the group which was bred there were 2 tumor mice and 19 without tumors in the F_2, compared with 10 tumors to 20 without in the backcross. The method of recording the production of young, which had been satisfactory for small groups of mice, unfortunately proved unreliable for large groups, so that there is a doubtful class in both F_2 and backcross mice. Among the F_2 in the doubtful group, there were

[9] Lathrop, A. E. C., and Loeb, L., The influence of pregnancies on the incidence of cancer in mice, *Proc. Soc. Exp. Biol. and Med.*, 1913, xi, 38; Further investigations on the origin of tumors in mice. III. On the part played by internal secretion in the spontaneous development of tumors, *J. Cancer Research*, 1916, i, 1. Loeb, L., VI. Internal secretion as a factor in the origin of tumors, *J. Med. Research*, 1919, xl, 477.

9 tumors to 49 non-tumors. If we classify these individuals in a manner least favorable to the theory (a manifestly unfair procedure) and regard all the tumor mice as having been bred and the non-tumors as not having been reproduced, there would be 11 tumors to 19 non-tumors in the F_2 group that was bred, compared with 10 to 20 in the backcross; and in the non-bred group 1 tumor to 58 non-tumor F_2 mice against 5 tumor to 15 non-tumor in the backcross. In all these



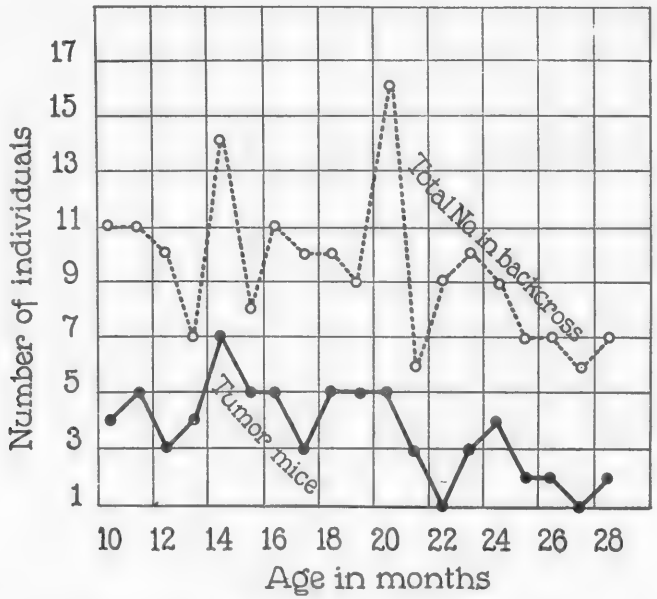
TEXT-FIG. 1. The age distribution of the F_2 individuals.

comparisons the greater number of tumors in the backcross as compared with the F_2 supports the theory that tumor susceptibility is inherited.

In any discussion of cancer rate, age distribution is a matter of importance, the conclusion reached above, however, cannot be discredited on the ground that the various groups were not comparable as to age. From Text-figs. 1 and 2 it will be seen that the ages cover much the same range. In the experiment as a whole, tumors appeared

at a lower age in the backcross than in the F_2. The comparison of the bred and non-bred groups appears equally valid considered from the point of view of age distribution as illustrated in Text-fig. 3.

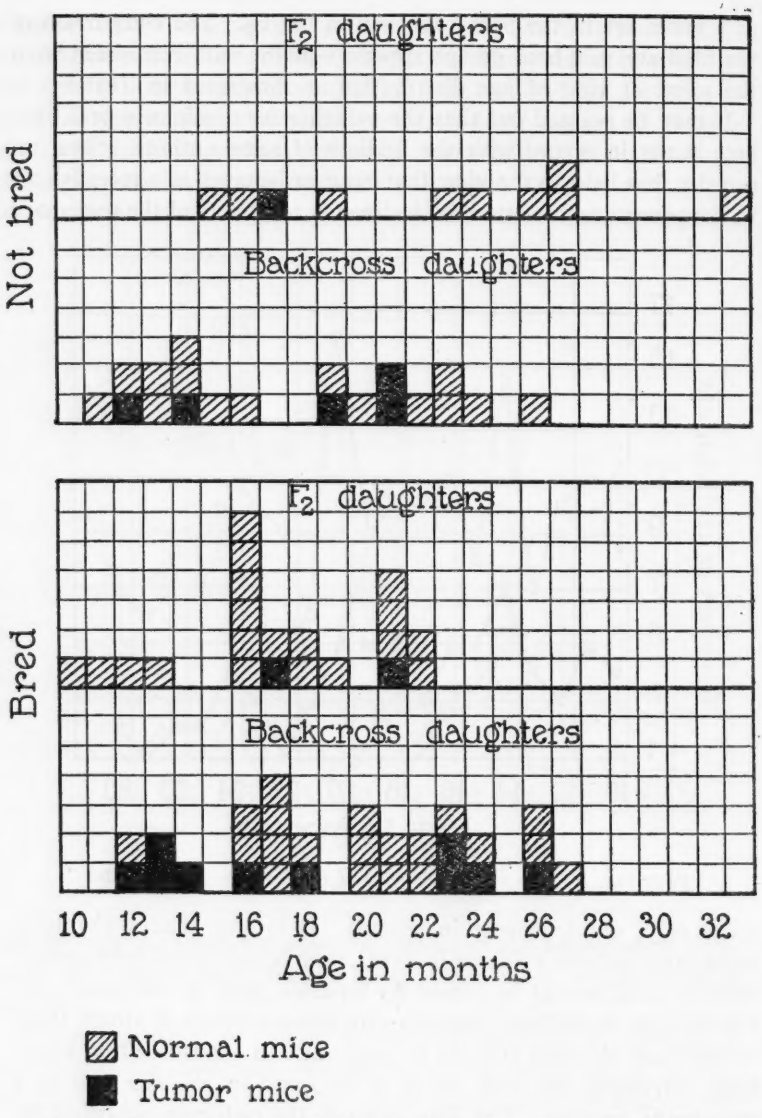
It may be pointed out that the evidence for dominance presented here is not in accord with the findings of some authors. Slye, especially, has held to the view that "cancer behaves as a recessive."[10] In the present report attention is directed to tumors of the mammary



TEXT-FIG. 2. The age distribution of the backcross individuals.

gland only, not to cancer in general. It is true that a character apparently uniform wherever it occurs, may in reality comprise several different entities and be caused by separate genes in the germ cell. For example, in chickens, there are four different kinds of whites, three of which are recessive and one is dominant. It is possible that mammary carcinoma also may act as a dominant in one race and as a recessive in another. The data given in the pedigrees published by

[10] Slye,[6] Eighteenth report, p. 76.



TEXT-FIG. 3. Comparison of the age distribution of tumor and non-tumor mice in the F_2 and backcross generations classified as bred and not bred.

Slye[6] includes 130 females with tumors of the mammary gland and the daughters obtained from them by either inbreeding or outcrossing to other (tumor?) stocks. Of these, 102 females had daughters with mammary gland tumor, while 28 females had daughters without such tumors. Of the 28 without cancerous daughters, the majority (24) had only 1 or 2 female offspring, but 1 mother had 3 daughters, 2 mothers had 4 daughters, and 1 had 5 daughters without cancer. There are apparently but two cases in which cancer stock has been crossed with absolutely non-cancer stock. In Strain 84, one cancerous female outcrossed to a male from non-tumor stock had one daughter and son without cancer. Slye calls this Branch II. This suggests the existence of additional offspring which were not reported. In Strain 164 a daughter of a cancerous mouse was outcrossed to a male from non-cancer stock and produced 4 sons and 4 daughters without tumor. Only individuals at least 6 months old (as we understand), are included in these charts, but the exact ages are not given, and since the average tumor age is much higher than 6 months and since the number of individuals reported is meager, these pedigrees do not present conclusive data. Possibly there are additional data not reported by Slye or incorrectly interpreted by us but the evidence, as published, seems inadequate to support the theory of recessiveness as applied to the mammary gland tumor.

CONCLUSION.

In this preliminary report there are presented some of the results obtained from crossing mice from tumor strains with males from other sources. The comparison of the tumor incidence in the inbred and backcross daughters, though the numbers given are small, supports the theory that the tendency to develop neoplasms is hereditary and the frequency with which tumors appear in the first filial generation of such crosses, indicates that the character is dominant.

Additional experiments involving larger numbers are in progress.